University of Alberta

Experimental Therapies for Pulmonary Arterial Hypertension

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the

requirements for the degree of *Doctor of Philosophy*

in

Experimental Medicine

Department of *Medicine*

Edmonton, Alberta

Spring 2007

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Your file Votre référence
ISBN: 978-0-494-29714-8
Our file Notre référence
ISBN: 978-0-494-29714-8

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“Success is the ability to go from one failure to another with no loss of enthusiasm.”

Sir Winston Churchill

Dedication

This thesis is dedicated to my mother, who taught me to never give up.

M. S. McMurtry

Abstract

Pulmonary Arterial Hypertension (PAH) is a disease that causes obliterative remodeling of the pulmonary arteries, leading to symptoms, right heart failure and death. There are few effective therapies and mortality is high. Traditionally, therapies for PAH have involved vasodilation of the pulmonary vascular bed, either by providing exogenous vasodilating substances such as calcium channel blockers, nitric oxide or prostacyclin and its derivatives, or by blocking vasoconstricting substances such as endothelin with endothelin receptor blockers. It is increasingly recognized that PAH involves remodeling of the pulmonary vasculature in addition to vasoconstriction, and that effective therapies for PAH must target this remodeling. We demonstrate that inhibition and reversal of the proliferative remodeling that leads to PAH is possible in a rat model by both drug therapy, with a metabolic modulator drug called dichloroacetate, and gene therapy, with a dominant negative survivin protein that promotes pulmonary artery smooth muscle cell apoptosis. Not all "anti-growth" or "pro-apoptotic" strategies are successful, and we demonstrate that drug therapy with combined sirolimus and atorvastatin, as well as gene therapy with human BMPR2, both fail to improve rat PAH. Therapies that target the proliferative remodeling of PAH, such as dichloroacetate or survivin inhibitors, may be beneficial in human PAH.

Acknowledgement

This work represents the combined efforts of many.

I thank my wife for her love and support, as well as delivering and minding our three wonderful children during the time these studies were performed.

I thank the other trainees in the lab, including Dr. Bernard Thebaud, Dr. Sebastien Bonnet and Rohit Moudgil for their collaboration, commiseration, and comeraderie.

I thank the technicians in the lab, including Kyoko Hashimoto, Gwynneth Harry, Alois Haromy, Sandra Bonnet, Xichen Wu, and Angie Hogan for all the help and instruction that they have provided.

I thank the staff in the Department of Medicine, including Dr. Dick Jones and Ms. Sharon Campbell, for their kind encouragement and help.

I thank Dr. Gary Lopaschuk for being a resource for both scientific procedural advice related to the completion of this work. I thank Dr. Peter Light, Dr. Lorne Tyrrell, Dr. Hank Duff, and Dr. Ken Weir for supervising my studies.

I thank Dr. Evangelos D. Michelakis for mentoring me these past few years. He has opened my eyes to the world of science, shown me a world of possibility, and proved that there is nothing that hard work cannot achieve. He has been uniformly kind yet firm, and has shown me by example not only how to be successful, but how to live with integrity. He has given more of his time and energy to me than any professor I have encountered before or since, and I shall be eternally grateful.

Finally, I thank Dr. Stephen L. Archer for mentoring me these past few years. More than anyone I have encountered in postgraduate training, he has believed in me and promoted me. He has been a role model for me as a cardiologist, as a scientist, as a leader, and as a family man. More than a supervisor, he has been a father figure for me. Words cannot express how much this has meant to me these past five years, and I cannot thank him enough. Unlike Isaac Newton, I may not have seen further than others, but I certainly have stood on the shoulders of giants.

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List of Abbreviations

5-HT, serotonin
5-HTT, serotonin transporter
ANOVA, analysis of variance
AOS, activated oxygen species
BMP, bone morphogenetic protein
BMPR2, bone morphongenetic protein receptor II
Ca++, calcium
CO, cardiac output
DCA, dichloroacetate
ET-1, endothelin-1
GFP, green fluorescent protein
iPAH, idiopathic PAH
K+, potassium
Kv, voltage gated K+ channel
LCM, laser capture microdissection
LVEDP, left ventricular end-diastolic pressure
MCT, monocrotaline
MnSOD, manganese superoxide dismutase
NIH, National Institutes of Health
NYHA, New York Heart Association
NO, Nitric Oxide
PA, pulmonary artery
PAAT, pulmonary artery acceleration time
PAH, pulmonary arterial hypertension
PASMC, pulmonary artery smooth muscle cell
PCNA, proliferating cell nuclear antigen
PHT, pulmonary hypertension
PPH, primary pulmonary hypertension
PPHN, persitent pulmonary hypertension of the newborn
PVR, pulmonary vascular resistance
PVRi, pulmonary vascular resistance index
qRT-PCR, quantitative reverse transcriptase polymerase chain reaction
RV, right ventricle
RVH, right ventricular hypertrophy
SMC, smooth muscle cell
TGF-β, transforming growth factor beta
VEGF, vascular endothelial growth factor
vWF, von Willebrand's factor
WHO, World Health Organization

Introductory Chapter

Pulmonary arterial hypertension (PAH) is a syndrome of elevated pulmonary arterial resistance, caused by a set of disorders with common pathobiological processes that lead to clinical presentations of dyspnea, chest pain, syncope, hemoptysis, and early mortality(3). Recognition of this syndrome postmortem began as early as 1891, when German physician Ernst von Romberg described "pulmonary vascular sclerosis" in autopsy specimens(113). In 1901, Dr. Abel Ayerza, an Argentinean physician, described a link between a clinical syndrome of dyspnea, cyanosis and polycythemia and sclerosis of the pulmonary artery. Dr. F.C. Arrilaga, a colleague, named the syndrome Ayerza's disease(39). In the 1930's, Dr. Oscar Brenner, an Englishman, correctly identified the clinical manifestations of Ayerza's disease as due to heart failure secondary to pulmonary disease(15). It was not until the 1960's before the link between the lesions of "pulmonary arterial sclerosis" and right ventricular hypertrophy was understood(39). The term Primary Pulmonary Hypertension (PPH), defined as PAH in the absence of an identifiable causative disorder, was originally coined by Dresdale in 1951(33) who made the first ante mortem diagnosis. PAH remained an obscure disorder until the 1960's, when an epidemic of PAH was detected that ultimate proved secondary to the anorexigen medication aminorex(41). A US National Institutes of Health sponsored PPH registry, originally designed to study the incidence and prevalence of PPH, subsequently demonstrated an association between anorexigen medications with PPH as well as valvular heart disease(25). The increased relative risk for valvular heart disease primarily, as well as for PPH, ultimately lead to the withdrawal of anorexigens from the commercial market. As a response to the epidemics of PAH associated with anorexigens in the 1960's the World Health Organization (WHO) convened a consensus conference in 1973(53). While this conference standardized terms and definitions of PPH, it was not until 25 years later, at a second WHO sponsored consensus conference in Evian, France, that the modern definition of PAH and classification system of all pulmonary hypertensive diseases, including secondary pulmonary hypertension, was developed and agreed upon(108). It should be made clear that pulmonary hypertension is not synonymous with PAH, and is a more general term that includes many diseases characterized by elevated mean pulmonary artery pressure including PAH but also pulmonary hypertension secondary to hypoxemia and lung disease, elevated left atrial pressure and heart disease, thromboembolic disease and other miscellaneous causes.

PAH definition and classification

The definition of pulmonary hypertension has been inherited from the US NIH PPH registry of the 1980's, which states that pulmonary hypertension is an elevated mean pulmonary artery pressure of 25 mmHg at rest or 30 mmHg with exertion(110). The 1998 WHO conference in Evian, France, divided all pulmonary hypertensive diseases into 5 categories that shared similar causes and approaches for therapy (Table 1). The first category is that of PAH, including familial and sporadic PPH as well as PAH associated with collagen vascular diseases, congenital systemic to pulmonary shunts, portal hypertension, drugs and toxins, and persistent pulmonary hypertension of the newborn (PPHN). The remaining categories are 2) pulmonary venous hypertension, generally but not exclusively associated with left heart disease, 3) pulmonary hypertension associated with hypoxia, generally due to intrinsic lung diseases, 4) pulmonary hypertension secondary to chronic thromboembolic disease, and 5) pulmonary hypertension associated with diseases that directly obstruct the pulmonary vasculature, such as schistosomiasis(108). While this classification scheme has been criticized for grouping disorders with different molecular and genetic causes, it has been found useful for clinical and epidemiologic purposes(39). The Evian classification was formally revised and updated at a third World Symposium at Venice in 2003(122), which also advocated the abandonment of the term PPH in favor of idiopathic PAH, or iPAH.

Table 1. The Evian Clinical Classification

1. Pulmonary arterial hypertension
    - 1.1 Primary pulmonary hypertension
        - a) Sporadic
        - b) Familial
    - 1.2 Related to
        - a) Collagen vascular disease
        - b) Congenital systemic-to-pulmonary shunts
        - c) Portal hypertension
        - d) Human immunodeficiency virus infection
        - e) Drugs/toxins
            - (1) Anorexigens
            - (2) Other
        - f) Persistent pulmonary hypertension of the newborn
        - g) Other
2. Pulmonary venous hypertension
    - 2.1 Left-sided atrial or ventricular heart disease
    - 2.2 Left-sided valvular heart disease
    - 2.3 Extrinsic compression of central pulmonary veins
    - 2.4 Pulmonary veno-occlusive disease
    - 2.5 Other
3. Pulmonary hypertension associated with disorders of the respiratory system or hypoxemia
    - 3.1 Chronic obstructive pulmonary disease
    - 3.2 Interstitial lung disease
    - 3.3 Sleep-disordered breathing
    - 3.4 Alveolar hypoventilation disorders
    - 3.5 Chronic exposure to high altitude
    - 3.6 Neonatal lung disease
    - 3.7 Alveolar-capillary dysplasia
    - 3.8 Other
4. Pulmonary hypertension caused by chronic thrombotic or embolic disease
    - 4.1 Thromboembolic obstruction of proximal pulmonary arteries
    - 4.2 Obstruction of distal pulmonary arteries
        - a) Pulmonary embolism (thrombus, tumor, ova, parasites or foreign material)
        - b) In situ thrombosis
        - c) Sickle-cell disease
5. Pulmonary hypertension caused by disorders directly affecting the pulmonary vasculature
    - 5.1 Inflammatory
        - a) Schistosomiasis
        - b) Sarcoidosis
        - c) Other
    - 5.2 Pulmonary capillary hemangiomatosis

PAH epidemiology

The prevalence, incidence, and natural history of PAH are incompletely understood, and complicated by the altered PAH definitions introduced by the Evian/Venice classification system. An unselected autopsy study of 17,901 subjects has estimated the prevalence of PPH at 0.13%(80), and early estimates of incidence in the USA was 1-2 cases of PPH per million per year(41). The US NIH PPH registry, which assembled a cohort of 187 patients with PPH, found that the median age at diagnosis is 36 years, with a mean time of symptom onset to diagnosis of 2 years and a ratio of female cases to male cases of 1.7:1(110). A subsequent study of patients from the NIH registry demonstrated a median survival of 2.8 years, with survival rates at 1, 3 and 5 years were 68%, 48% and 34% respectively. Mortality increased with advanced NYHA class, elevated right atrial and mean PA pressures and decreased cardiac index(29). Similar findings were observed in studies of retrospective cohorts in Israel(2), the UK(97), France(16), India(106) and Japan(98). The annual mortality burden of PPH in the USA has been increasing over the last thirty years(61, 72), but it is not certain whether this increase reflects an increased incidence in the disease versus improved detection and recognition of cases. A recent retrospective study of 84 patients with iPAH or anorexigen-associated PAH showed survival rates at 1, 2, and 3 years of 87%, 75%, and 61%, respectively(65). Being of African-American or Asian descent was independently associated with an increased risk of death, which may be attributable to biologic or socioeconomic differences. Warfarin use, higher cardiac index, and acute pulmonary vasoreactivity were protective.

Less is known about the epidemiology of non-idiopathic PAH. Estimates of PAH in the setting of connective tissue disease, predominantly systemic sclerosis, range from 12 to 27%(92, 105, 142), and appears to be an indicator of poorer prognosis(128). PAH commonly complicates congenital heart disease, particularly those with pressure and volume overload. However, there are no epidemiologic studies that accurately estimate the incidence, prevalence, and outcomes of PAH associated with these congenital heart lesions, though the long-term survival seems superior to other forms of PAH. The incidence of PAH in patients with HIV is estimated at 0.1% per year(101), which several hundred-fold increased over the general population. PAH in the setting of HIV appears to improve with treatment of the HIV infection with combination anti-retroviral therapies(150), but is associated with significant morbidity and mortality necessitating treatments for PAH as well(96).

PAH Histopathology

The nomenclature and schema for describing the histopathology of PAH has been standardized(103). Lesions include non-specific changes that occur in all forms of pulmonary hypertension, such as medial hypertrophy of muscular and elastic arteries, dilatation and intimal atheromas of elastic pulmonary arteries (PAs), right ventricular hypertrophy, as well as PA medial and intimal thickening. However, the pulmonary circulation is relatively resistant to atheroma formation. What characterizes PAH are constrictive and complex pre and intra-acinar angioproliferative plexiform lesions that are not observed in other forms of pulmonary hypertension.

The obstructive lesions include medial hypertrophy, intimal thickening, and adventitial thickening, and are thought to be due to an imbalance between apoptosis and proliferation of the cells that comprise theses layers of the PAs. They are typically diffuse lesions, and likely increase pulmonary vascular resistance both by causing luminal loss and active vasoconstriction. Medial hypertrophy is an increase in the cross sectional area of vessel wall, and is caused by hypertrophy and hyperplasia of smooth muscle cells and an increase in connective tissue and extra cellular matrix, with muscularization of arterioles extended further into the normally non-muscularized arterioles. Intimal thickening, resulting from proliferation of smooth muscle cells, fibroblasts, and myofibroblasts, and may be concentric laminar, concentric non-laminar, and/or eccentric. Adventitial thickening is difficult to define and quantify, as the boundary of adventia and media is often not sharply defined. Increased connective tissue and extra cellular matrix build-up is common (Figure 1-1).

Complex lesions include plexiform lesions, dilation lesions, and arteritis, and are focal changes that may indicate PAH severity or rapid progression. Plexiform lesions are obstructing, glomerulus-like collections of endothelium channels that are lined by myofibroblasts and smooth muscle cells, and have been proposed as a clonal tumor-like structure(70). The dilation lesion is a thin-walled structure not unlike a vein, which is susceptible to pulmonary hemorrhage, in-situ thrombosis, and fibrotic organization. Arteritis, with infiltration of the vascular wall with chronic and acute inflammatory cells, is rarely primary in PAH but occasionally seen. Interestingly, all these lesions may be seen in a single patient.

PAH pathobiology

PAH is characterized by elevated pulmonary vascular resistance (PVR), which is due to the processes of vasoconstriction, vessel remodeling, and vessel obstruction due to thrombosis, though the relative contribution of each are not equal. The increased right ventricular afterload reduces cardiac output, causes symptoms of fatigue, dyspnea, edema, and syncope, and ultimately leads to right heart failure and death. It is increasingly realized that the predominant processes that elevates PVR is remodeling of the resistance PAs(62). Important derangements that may be pathogenic have been described at each layer of the vascular wall from lumen to adventitia. These derangements include abnormal endothelial cell growth and function, with increased endothelial derived vasoconstrictors and mitogens(49) and decreased endothelial derived vasodilators(48, 132); abnormal growth and proliferation of pulmonary artery smooth muscle cells with abnormal phenotypes(60), including reduced K+ channel expression(145, 146); abnormal platelets with decreased levels of serotonin (5-HT) in the platelets and increased levels in the serum(58, 76); abnormal activation of proteases within the vessel wall, and a procoagulant state(3).

The endothelium. The endothelium in PAH is highly abnormal(18). Because PAH is usually detected only late, there are no studies of endothelial function early in the course of the disease. It has been postulated, however, that endothelial cell injury and loss, are mechanistically important early in the disease due both to loss of endothelial derived vasodilating substances, such as NO, and exposure of the subintimal tissues to mitogenic substances within the plasma(85, 149). Prostacyclin, an endogenous endothelial-derived vasodilator substance that is also antiproliferative, is reduced in PAH(132) and there is an imbalance between vasodilating prostacyclin and vasoconstricting thromboxane A2, possibly contributing to a vasoconstricted state in PAH(24). Similarly, endothelial nitric oxide synthase (eNOS) expression is reduced in PAH, possibly contributing to a vasoconstrictive and proliferative state(48). Another vasodilating substance, vasoactive intestinal peptide, is also reduced in PAH(100). Endothelin-1 (ET-1) is a potent vasoconstrictor and mitogen, and levels of this substance, some of which is synthesized in the lungs, are increased in PAH(49). The net effect of these deficiencies of vasodilators and increased constrictors is to shift the balance of tone to a more vasoconstricted state, resulting in increased PVR but also stimulating vascular cell proliferation(3). Some of these abnormalities, notably increased endothelin-1are seen also in other forms of pulmonary hypertension, and therefore are not the primary causes of PAH. The endothelial dysfunction appears to be a response to injury. Nonetheless, replacement of reduced vasodilating substances and blockade of vasoconstrictors makes up the cornerstone of current therapy for PAH(5).

An additional and intriguing abnormality of the endothelium that has been described in PAH is clonal expansion of endothelial cells within plexiform lesions(70). This observation suggests that the proliferative remodeling of the vascular wall in PAH might be a form of neoplasia. Possibly early endothelial damage selects an apoptosis resistant and pro-proliferative clone that ultimately populates and obstructs the pulmonary vasculature in PAH. This notion is controversial, but deserves further study.

K+ channels. K+ channels are transmembrane proteins that contain a central ion pore with selectivity for K+. They are central to the maintenance of cellular membrane potential(4). K+ channels are tonically active in PASMCs, and the slow efflux of K+ down they intracellular/extracellular K+ gradient (K_o/K_i = 140/5mM) contributes to keeping the PASMC membrane hyperpolarized (~-60mV). K+ channels modulate vascular tone. Inhibition or loss of K+ channels leads to SMC depolarization, activation of L-type calcium channels, and an intracellular influx of calcium and activation of the SMC contractile apparatus as well as proliferation. There are many types; K+ channels have 4 classes, including voltage-gated (Kv), ATP sensitive K+ channels, inward rectifier (Kir) and calcium-sensitive K+ channels. Beyond maintenance of SMC membrane potential and regulation of vascular tone, K+ channels may also modulate apoptosis. Increased intracellular K+ due to K+ inhibition or loss inhibits several caspases involved in apoptosis(107), and therefore decreased K+ current due to loss or dysfunction of K+ channels might lead to not only a vasoconstricted but a proliferative state. The relative contribution to PAH from decreased K+ current due to vasoconstriction directly, proliferation caused by increased intracellular calcium, and decreased apoptosis, is unknown.

In humans with iPAH, Kv1.5 expression is reduced and available channels are dysfunctional with reduced current(145, 146), and in turn there is PASMC membrane depolarization and increased intracellular calcium. Furthermore, the anorexigens fenfluramine, dexfenfluramine, and aminorex that have been associated with epidemics of PAH(112) are potent Kv channel blockers(138). Kv1.5 is decreased in monocrotaline PAH, chronic hypoxic pulmonary hypertension, and in the fawn hooded rat model of pulmonary hypertension, while other K+ channel expression is unchanged(14, 83, 87). These data suggest that the selective downregulation of Kv channels might play a key role in the pathogenesis of PAH(3), making PAH a form of "channelopathy."

Serotonin. Serotonin (5-hydroxytryptamine, 5-HT) has been shown to be a pulmonary vasoconstrictor in dogs(81), and a mitogen for bovine PASMCs in culture(71). Platelets, which are the main storage pool for 5-HT, have reduced 5-HT levels in PAH while serum 5-HT levels are increased(58). Furthermore, PAH has been observed in some cases of platelet 5-HT storage disorders(57). Aminorex and the fenfluramines, the anorexigens associated with epidemics of PAH(112), have been associated with inhibition of platelet reuptake of 5-HT by interacting with the 5-HT transporter (5-HTT)(76). It has been recently shown that 5-HTT is overexpressed, primarily in the PA media, in patients with PAH, and that this is associated with abnormally increased PASMC growth when stimulated by 5-HT or serum(37). 5-HTT is encoded by a single gene in chromosome17q11.2, and the L-allelic variant of the 5-HTT gene promoter, which induces a greater rate of 5-HTT gene transcription than the S allele, has been reported by one group to be associated with 5-HTT over-expression in PAH (65% of PAH patients but in only 27% of controls)(37), though this has not been observed in all studies(144). Mice over-expressing the 5-HTT gene exhibit spontaneous PHT in normoxia and exaggerated PHT in hypoxia(75). On the other hand, mice lacking the 5-HT1B receptor develop less PHT in response to hypoxia than controls(66), while mice lacking the 5-HT2B receptor, which may regulate plasma 5-HT levels(20), do not develop any hypoxic

PHT(69). These data suggest that abnormalities of serotonin handling, either genetic or acquired, might be causative of PAH.

A link between K^+ channels, anorexigens and 5-HT has been proposed. The anorexigens block Kv channels in platelet progenitor cells, megakaryocytes, leading to 5-HT release(139). Furthermore, fenfluramine reduces Kv1.5 mRNA levels by 50% in PASMCs from normotensive patients(135), suggesting that inhibited gene transcription and expression of Kv channels may play an important role in anorexigen-PAH. These data suggest implicate the lack of Kv channels in platelets or pulmonary endothelial cells in the increase of 5-HT seen in PAH.

TGF-β Superfamily. A genetic cause for PAH has long been suspected based on the grouping of PAH cases in families. Mutations of the bone morphogenetic receptor II (BMPR2) occur in ~75% of patients with familial PAH(31, 68), although they appear to be relatively rare in sporadic PAH (~10%) as well(93). A member of the TGF-β superfamily, BMPR2 is active in embryologic growth and development(11). BMPR2 is a constitutively active serine-threonine kinase receptor that, in response to ligand (BMP2, 4, 7), forms hetero-dimers with any of 3 type 1 receptors (BMPR1A, BMPR1B or Activin receptor-like kinase (Alk2). This association results in phosphorylation of the intracellular portion of the type 1 receptor by BMPR2, initiating a cytosolic Smad protein signaling cascade(120). Receptor activated (R-SMADs), including Smad 1, 5 and 8, complex with common partner SMAD (Smad4) permitting it to translocate to the nucleus where it can regulate gene transcription. The Smad DNA interaction is weak and requires co-repressors or activators. In the nucleus, R-Smad/co-Smad complexes interact with genes that have a Smad-binding element (5-AGAC-3). This binding alters PASMC proliferation and apoptosis. BMPR2 receptors are expressed in human PASMC and their activation by BMPs leads to phosphorylation of SMAD1 and apoptosis, associated with decreased expression of Bcl(148).

Reduced pulmonary vascular BMPR2 expression has been described in human PAH(6), and PASMCs from patients with PAH proliferate abnormally in response to BMP ligands(91). Mice with haploinsufficiency of BMPR2 have mild PAH (10, 140), and are more susceptible to PAH when exposed to pulmonary vascular stressors(73, 124). Discoveries of related TGF-β superfamily members that are associated with PAH and hereditary hemorrhagic telangiectasia, such as ALK 1(129) and endoglin(23), have also strengthened the case for BMPR2 playing an important role in the development of PAH. Interestingly, abnormalities on the TGF-β-BMP pathways have been described in tumors, like the juvenile colonic polyposis(119), or vascular lesions such as the coronary restenosis lesions post angioplasty(79). These studies implicate deficient BMPR2 in the development of PAH.

Angiogenesis. Plexogenic lesions, the complex bundle of endothelial cell channels that have dysregulated expression of several angiogenesis related proteins, including vascular endothelial growth factor (VEGF), VEGF receptor 2, PI3 Kinase, src, and Akt(131). Based on these findings, disordered angiogenesis has been implicated in the pathogenesis of PAH and the formation of plexogenic lesions(3). Most PAH patients have systemic hypoxemia and reduced alveolar diffusion capacity, however, even though their alveolar PO_2 is normal. Plexogenic lesions may therefore not be a primary abnormality in PAH but rather a secondary phenomenon, caused by a disordered angiogenesis in response to local tissue hypoxia, distal to the obliterated pulmonary arteries.

The role of VEGF in the pulmonary circulation in incompletely understood, but it has been proposed that VEGF is involved in endothelial cell maintenance and survival(60). VEGF exists in at least two isoforms, with VEGF-A thought to be protective in PAH and VEGF-B potentially pathogenic. VEGF-A, when given by cell based gene transfer, has been demonstrated to ameliorate rat monocrotaline PAH(21), possibly by preserving distal resistance PA lumen. In contrast, VEGF-B deficient mice have reduced pulmonary hypertension when exposed to hypoxia(136). Blockade of VEGF receptor 1 (VEGFR-1) and chronic hypoxia has been associated with endothelial cell dysfunction and cell death, suggesting the injury and apoptosis of endothelial cells may contribute to the pathogenesis of PAH(127).

Additional growth factors, including platelet derived growth factor, basic fibroblast growth factor, insulin-like growth factor-1, and epidermal growth factor may also be important in the remodeling of PAH, though there is little data(60). Angiopoietin-1, another angiogenic factor, has also been studied in PAH, but the data are conflicting. It has been suggested that all forms of non-familial pulmonary hypertension are characterized by upregulated angiopoietin-1, which in turn correlates with PVR(34, 126). In contrast, other investigators have found a protective role for angiopoiten-1 in experimental PAH, with angiopoiten-1 gene therapy reducing monocrotaline PAH in rats, possibly by reducing endothelial cell loss and endothelial dysfunction(149). More data are required to make sense of this apparent contradiction.

Proteolysis. Breakdown of extra-cellular matrix by vascular serine elastases and matrix metalloproteinases exposes vascular SMCs to matrix-bound mitogens as well as induces the formation of mitogenic tenascin C(28). Inhibition of serine elastases and matrix metalloproteinases can reverse PASMC proliferation and PA remodeling in vitro. Serine elastases have been shown to increase matrix metalloproteinase activity, as well as antagonize inhibitors of matrix metalloproteinases, and have been shown to precede activation of matrix metalloproteinases in some animal models of vascular injury(60). Furthermore, inhibition of serine elastases with experimental compounds (M249314 or ZD0892) has been shown to reverse established rat monocrotaline pulmonary hypertension(27). Augmented serine elastases activity in PAH might be in part due to reduced bioavailability of nitric oxide, which reduces serine elastase activity via cGMP-mediated suppression of ERK phosphorylation and AML1B nuclear partitioning(89).

Research in the last decade has yielded an explosion of insight into the mechanisms of pulmonary vascular remodeling and PAH, though these processes and their relationships remain incompletely understood. It is apparent that most of these abnormalities result in either enhanced vasoconstriction, impaired apoptosis or accelerated proliferation of the vascular cells. Likely no one mechanism explains PAH, and it may be necessary for there to be a "multiple hit" of both genetic and environmental factors to develop PAH, not unlike many forms of cancer(3). The phenotype of PAH may indeed be a "final common pathway" of response to injury, with many possible causes attributing to any one case(74). It is hoped that research will identify additional therapeutic targets and lead to new treatments that reverse the proliferative PA remodeling that characterizes PAH.

Current PAH therapies

Conventional medical therapy for PAH begins with a set of standard instructions for care, such as avoiding physiologic stressors including pregnancy, altitude, and vigorous aerobic exercise(5). The evidence for these measures is consensus, as opposed to randomized clinical trials. Similarly, supplemental oxygen is often provided for symptom relief but this measure is unproven(44).

Anticoagulants. Oral anticoagulation with coumadin has been used for many years based on the pathologic observation of in-situ thrombosis in tissues specimens of PAH. Retrospective single center studies of coumadin do demonstrate a 50-100% increase in survival in those PAH patients that received coumadin(40, 111), but because treatment decisions were not randomized the result is subject to bias. There is no evidence to suggest that the possible benefit is restricted to patients with worse functional capacity. Based on these studies, PAH patients are often anticoagulated.

Diuretics. Diuretic therapy is often used to control the symptoms of right heat failure, such as peripheral edema. Because these agents are clearly beneficial in reducing edema, there have been no randomized studies performed. However, there is no evidence that they affect mortality or the progression of PAH and it is important to not reduce left ventricular end diastolic pressure, which can cause hypotension. Similarly, digoxin is often administered to PAH patients with right heart failure to ameliorate symptoms. Digoxin is weakly inotropic, and can increase cardiac index in the short term(44). No randomized studies have been performed which demonstrate long-term efficacy in symptom reduction or improved survival, and the treating physician does digoxin administration empirically.

Calcium channel blockers. Calcium channel blockers have been used as therapy for PAH since the 1980's to attempt to vasodilate the pulmonary arterial bed. In a non-randomized prospective study of 64 patients with iPAH, 26% responded acutely to high-dose calcium channel blockers with an acute decrease in pulmonary vascular resistance (PVR) of 20%(109). Patients that responded, and who could tolerate long-term therapy experienced a markedly improved survival at 5 years versus non-responders (94% vs. 55%). Since the patients given calcium channel blockers were not randomized but selected by having acute pulmonary vasodilation, it may be that the improved survival is due to unexplained differences in the iPAH mechanism, or stage disease at presentation instead of the calcium channel blocker therapy. Regardless, in practice fewer than 20% of patients with PAH have a significant reduction in PVR with acute challenge of pulmonary vasodilators, such as calcium channel blockers, epoprostenol, or inhaled nitric oxide, and only half of those will experience enduring reductions in PVR or improved functional capacity(123). Furthermore, there is no evidence that demonstrates benefit in forms of PAH besides iPAH. Nonetheless, it is conventional to perform vasodilator trials in other forms of PAH and prescribe calcium channel blockers to responders.

Prostacyclin. PAH is characterized by a decrease in endothelial derived vasodilating prostaglandins (prostacyclin)(24), which is the rationale for prostacyclin analogue therapy in PAH. The efficacy of continuous IV infusions of synthetic prostacyclin (Flolan), or epoprostenol, has been studied in two randomized trials of New York Heart Association (NYHA) class III and IV patients with iPAH(9, 115) and one randomized trial of PAH associated with systemic sclerosis(7). All three trials demonstrated improved functional capacity, with increases in the six minute walk between 100 to 150 meters, as well as modest reductions in mean PA pressure. A short term survival benefit was observed in one study(9), with 8 deaths in the control group versus none in the treatment arm after 12 weeks of follow-up. While the improvements in hemodynamics, six minute walk, and mortality are at best modest, and the study sizes are small and of limited duration, epoprostenol remains the cornerstone of therapy for PAH and is the only treatment with any evidence of survival benefit. Limitations of epoprostenol include the need for continuous IV infusion via an indwelling catheter, with concomitant risk for line complications, as well as significant expense (>$60,000 per year)(5).

Prostacyclin analogues. Because epoprostenol must be given as a continuous infusion, which necessitates an indwelling catheter, synthetic prostacyclin analogues that can be given by alternate routes have been developed. Treprostinil is given by subcutaneous injection, and has been studied in two randomized clinical trials of patients with PAH(82, 121). Treprostinil has been shown to reduce PVR by approximately 20%, and is associated with very modest increases in six minutes walk of 16-37 metres. Beraprost is an orally active prostacyclin analogue, and has been studied in two randomized controlled trials of NYHA class II and III patients with PAH(8, 43). Modest improvements were demonstrated in six minute walk (20-30 metres) at three and six months, but these improvements were not sustained at one year(8). Iloprost, available in Europe but not North America, is a prostacyclin analogue that can be inhaled. It has been studied in one randomized controlled trial that enrolled NYHA class III and IV patients with PAH as well as chronic thromboembolic pulmonary hypertension(99). The patients with iPAH responded best, with a median increase in six minute walk of 58 metres over 12 weeks. These prostacyclin analogues are modest effective at improving hemodynamics and functional capacity in PAH, and therefore used as an alternative to IV epoprostenol in some patients. Furthermore, the studies of prostacyclin analogues remind us that not all forms of PAH are the same in terms of response to therapy or survival.

Endothelin receptor blockers. Endothelin is a potent arterial vasocontrictor and mitogen that is elevated in PAH(125). Bosentan is a non-selective endothelin receptor blocker that has been studied with a randomized controlled trial in patients with NYHA class III and IV iPAH and PAH due to systemic sclerosis. Bosentan is an oral medication that has been shown to improve six minute walk with a mean difference between the treatment and placebo group of 44 metres. Bosentan improves functional class and increases the time to clinical worsening(114). Significant limitations to Bosentan therapy include the cost, as well as rates of hepatotoxicity of approximately 10%(5). Sitaxsentan is an endothelin receptor Type A specific blocker, which has the theoretical advantage over Bosentan of blocking the Type A mediated vasocontrictive and mitogenic properties, while leaving intact the Type B mediated NO dependent vasorelaxation(5). Sitaxentan has been studied in a randomized controlled trial of NYHA class II, III and IV patients with iPAH or PAH due to systemic sclerosis or congenital heart disease(141). Six minute walk was increased by 35 metres at 12 weeks, and NYHA functional class increased in 29% of patients. No dose effect was observed, but significant hepatotoxicity was observed at higher doses (300mg vs. 100mg daily). Like most studies of PAH, it was not powered to test for a survival benefit.

Phosphodiesterase inhibitors. Phosphodiesterase-5 is preferentially expressed in the pulmonary and genital circulations. This enzyme rapidly degrades cyclic-GMP. Inhibition of phosphodiesterase-5 causes pulmonary arterial vasodilation(84, 88) as well as having an anti-proliferative effect on the pulmonary circulation(5). Sidenafil has been shown to increase six minute walk in patients with iPAH and PAH due to systemic sclerosis or congenital heart disease by 51 metres, an effect that was durable at one year(42). No mortality benefit was observed. Sildenafil has no significant hepatotoxicity, and is much less expensive than prostacyclin analogues or endothelin receptor blockers(5). A small trial of 26 patients with PAH, the SERAPH study, compared sildenafil and bosentan, and found no significant difference in six minute walk between the two groups at sixteen weeks(143). This trial may have been underpowered, as sildenafil improved six minute walk by 114 metres [67, 160] compared to 59 metres [29, 89] for bosentan. One death was observed in the sildenafil group, though it is not clear that it was due to sildenafil therapy. Furthermore, only sildenafil reduced right ventricular hypertrophy.

Combination therapy. Similar to therapeutic strategies in the management of HIV, tuberculosis, and cancers, there is great interest in combining therapies in the search for increased and synergistic therapeutic effect. Trials of combination therapies are being performed(47, 50, 59), though it is early in the evaluation of multi-drug strategies and the optimal combinations of treatments is not clear. The most promising strategy to date is the combination of iloprost and sildenafil(47).

Lung transplantation and atrial septostomy. Select patients that are NYHA class III or IV and have failed all medical therapy may be offered lung transplantation(67). Lung transplantation for PAH has not been studied in randomized trials due to the unethical nature of randomization when no alternative treatment exists. Survival rates post lung transplant for PAH are 55% at three years and 45% at 5 years(56).

Although unproven in prospective or randomized trials, atrial balloon septostomy is occasionally offered as a bridge to lung transplantation in PAH patients that are transplant candidates and have failed medical therapy(67).

Interim Summary

PAH is a group of related disorders with similar clinical presentations, similar treatment strategies, and related pathobiologies that are incompletely understood.

Patients with PAH have a high burden of morbidity and early mortality. Traditional therapies for PAH, which are predominantly pulmonary vasodilators, are at best only modestly effective and despite state of the art therapy many patients suffer and face early mortality. Minimal improvements in six minute walk of 50 metres or less come at the cost of thousands of dollars per year, as well as significant medication side effects. Recent advances of understanding of the biology of PAH have ushered in a new paradigm of therapeutic strategies, targeting the vascular remodeling that characterizes PAH. This idea of targeting the abnormal growth and proliferation of cells in the resistance pulmonary arterial bed to prevent or reverse the remodeling of PAH, and induce corresponding improvements in hemodynamics, morbidity, and mortality, is the central hypothesis of this work.

Targeting the mitochondria: mitochondria-induced apoptosis in PAH

Vascular medial remodeling results from an imbalance between smooth muscle cell (SMC) proliferation and apoptosis, favoring proliferation. Gene microarray studies show that lungs from patients with PAH have a decrease in the proapoptotic/antiapoptotic gene expression ratio(45). Furthermore, several loss-of-function germline or acquired mutations have been described in receptors of the transforming growth factor-β superfamily, such as bone morphogenetic protein receptor-2 (BMPR-2), in patients with primary PAH(116). Activation of the transforming growth factor-β/BMPR2 axis suppresses proliferation and activates apoptosis in normal PA smooth muscle cell (PASMC)(148) but not in PASMC from patients with PAH(91). This resistance to apoptosis is further enhanced by the selective downregulation of Kv channels that has been shown in human(45, 146)and animal models of PAH(87) Intracellular K+ levels, which increase when K+ channels are inhibited or downregulated, exhibit a tonic inhibition of caspases in many cell types, including PASMC(107). In addition to a suppression in apoptosis, Kv channel downregulation leads to PASMC depolarization, opening of the voltage-gated Ca++ channels, and increased intracellular Ca++; in turn, this causes vasoconstriction and increased PASMC proliferation(104).

The role of mitochondria in pulmonary vascular biology and PAH is unknown. However, mitochondria are potentially important because they regulate both apoptosis (by the release of proapoptotic factors, including the caspase activator cytochrome c(147)) and vascular tone (by the production of activated oxygen species [AOS]). AOS can "leak" to the cytoplasm and affect redox-sensitive second messenger systems and membrane K+ channels. For example, superoxide is produced in the proximal electron transport chain (ETC) and, in the presence of the mitochondria-based manganese superoxide dismutase (MnSOD), it is dismutated to H_2O_2, a well-characterized K+ channel opener and vasodilator(19, 86). Therefore, vascular mitochondria might be important targets for the treatment of vascular disease.

Anti-Survin Therapy for PAH

Survivin, a member of the mammalian "inhibitor of apoptosis" family(118), is known to be expressed in essentially all cancers but not in most normal adult cell types(1). The cell cycle–dependent expression of the survivin gene in mitosis suggests a role for survivin in promoting cell proliferation; however, recent data point to a more selective role of survivin in antagonizing mitochondria-dependent apoptosis, and a mitochondrial pool of survivin has recently been shown in cancer cells(32). The absence of survivin from most healthy tissues makes it very attractive as a target for therapy. Molecular antagonists of survivin, including antisense and dominant-negative mutants, have been consistently associated with induction of apoptosis and inhibition of tumor growth in vivo, without affecting normal cells(1). Such a mutant is the Thr34→Ala (here described as survivin-M). This mutation prevents a critical phosphorylation of endogenous survivin by the mitotic kinase p34cdc2–cyclin B1. Survivin-M has a 4- to 5-fold accelerated clearance compared with WT survivin and results in a dominant-negative effect by lowering endogenous survivin levels(1). In addition to inducing apoptosis in cancer cells(1), survivin targeting with survivin-M prevents vascular remodeling in an arterial injury model by inducing apoptosis within the vascular wall(13). In that study, survivin was absent in quiescent SMCs but was induced in vitro by exposure of aortic SMCs to 20% serum or PDGF or selectively in injured arterial segments in vivo(13). The pulmonary circulation is very different from the systemic circulation; for example, the pulmonary circulation has low pressure compared with the systemic circulation and constricts to hypoxia, while the systemic circulation dilates(137). This difference might be in part due to the fact that PASMC mitochondria, important oxygen sensors, are different from the systemic arterial SMC mitochondria: they have lower respiratory rates, are more depolarized, have more manganese superoxide dismutase (MnSOD), and produce more hydrogen peroxide(86). The mitochondria-produced hydrogen peroxide can activate both Kv channels(22, 134) and guanylate cyclase(19), thereby causing pulmonary vasodilatation. By controlling both vascular tone and apoptosis(35), mitochondria are potentially important in the etiology and therapy of vascular disease, but their role in PAH is not known. By targeting expressed survivin in the pulmonary arterial tree in PAH, such as with a dominant negative form like survivin-M, mitochondrial-dependent apoptosis may be augmented resulting in reduction resistance PA remodeling and less PAH.

Rapamycin and Statins for PAH

Rapamycin is an immunosuppressant originally isolated from the bacterium *Streptomyces hygroscopicus*(133). Rapamycin binds to a intracellular receptor called FKBP12(12), and the rapamycin-FKBP12 complex binds mTOR (mammalian target of rapamycin)(17, 55, 117), a ~280 kDa serine/threonine kinase(36). This rapamycin-FKBP12-mTOR complex activates S6 kinase, which phosphorylates S6 (a 40S ribosomal protein) and in so doing modulates the translation of ribosomal proteins and translation elongation factors(130), arresting cells in the late G1 phase of the cell cycle(36). Rapamycin has clinical utility in transplant medicine as an immunosuppressant(63), in cardiovascular medicine as an antiproliferative agent to reduce in-stent restenosis(90), and has promise as an anti-cancer agent(102).

Recently, Nishimura et al reported that rapamycin attenuates PAH and suppresses neo-intimal proliferation in a model induced the combination of pneumonectomy plus monocrotaline (MCT, 60mg/kg) (95), a variant of the monocrotaline model of PAH. Interestingly, this study found that rapamycin failed to reverse established PAH, which, if verified, would be an important practical limitation of such a strategy. In addition, activation of mTOR is necessary for pulmonary artery adventitial fibroblast proliferation(46) which accounts for hypoxia-induced adventitial remodeling in the chronic hypoxia model of pulmonary hypertension.

Simvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor ("statin") that is used widely in the primary and secondary prevention of atherothrombosis(51, 78). Simvastatin, as well other statins, are postulated to have beneficial "pleiotropic" effects on the vasculature beyond LDL reduction(26, 30), including augmentation of endothelial function, nitric oxide mediated vasodilation and inhibition of proliferation of endothelial and vascular smooth muscle cells. Simvastatin has been shown in one study to reverse established MCT PAH in rats by inducing apoptosis of neo-intimal smooth muscle cells(94). Although there are no randomized human trials of simvastatin in PAH, it is being used in some patients at some centers and a positive observational series has been published(64). Furthermore, there is a clinical trial, based at Imperial College London, currently randomizing human patients with PAH to simvastatin or placebo (ClinicalTrials.gov Identifier: NCT00180713). Atorvastatin is a more potent statin than simvastatin, in terms of reducing cholesterol and cardiovascular endpoints(52), and may have more potent pleiotropic effects, such as enhancement of endothelial derived nitric oxide availability, suppression of inflammation, and inhibition of oxidative stress, as well(77).

BMPR2 Gene Therapy for PAH

A genetic cause for PAH has long been suspected based on the occasional grouping of PAH cases in families. Mutations of the bone morphogenetic receptor II (BMPR2) occur in ~75% of patients with familial PAH (31, 68), although they appear to be relatively rare in sporadic PAH (~10%) as well(93). A member of the TGF-β superfamily, BMPR2 is active in embryologic growth and development(11). BMPR2 is a constitutively active serine-threonine kinase receptor which, in response to ligand (BMP2, 4, 7), forms hetero-dimers with any of 3 type 1 receptors (BMPR1A, BMPR1B or Activin receptor-like kinase, Alk2. This association results in phosphorylation of the intracellular portion of the type 1 receptor by BMPR2, initiating a cytosolic protein signaling cascade consisting of Smad proteins(120). Receptor activated (R-SMADs), including Smad 1, 5 and 8, complex with a common partner SMAD (Smad4) permitting it to translocate to the nucleus where it can regulate gene transcription. The Smad DNA interaction is weak and requires co-repressors or activators. In the nucleus, R-Smad/co-Smad complexes interact with genes that have a Smad-binding element (5-AGAC-3), altering PASMC proliferation and apoptosis. BMPR2 receptors are expressed not only in human endothelium but also in human PASMCs and their activation by BMPs leads to phosphorylation of SMAD1, decreased Bcl expression and apoptosis(148).

Reduced pulmonary vascular BMPR2 expression has been described in human PAH(6), and PASMCs from patients with PAH proliferate abnormally in response to BMP ligands(91). Mice with haploinsufficiency of BMPR2 have mild PAH(10, 140), and may be more susceptible to PAH when exposed to pulmonary vascular stressors(73, 124). Discoveries of related TGF-β superfamily members that are associated with PAH and hereditary hemorrhagic telangiectasia, such as ALK 1(129) and endoglin(23), have also strengthened the case for BMPR2 playing an important role in the development of PAH. Interestingly, abnormalities on the TGF-BMP pathways have been described in tumors, like the juvenile colonic polyposis(119), or vascular lesions such as the coronary restenosis lesions post angioplasty(79). Based on the evidence implicating deficient BMPR2 in the development of PAH, overexpression of BMPR2 in the pulmonary vascular tree using an adenoviral gene therapy is a reasonable therapy to try in experimental PAH.

PAH Animal Models

There are at least three common and accepted rodent models of acquired pulmonary hypertension. These include the monocrotaline model, the chronic hypoxia model, and the fawn-hooded rat model. Monocrotaline, an extract from the weed crotalaria spectabilis, induces upon single injection hemodynamic and morphometric changes in the pulmonary circulation that very closely resemble human PAH(54). While monocrotaline can induce pulmonary hypertension in many animals, it is the rat model that is the most convenient to study in most instances. Variations on the model exist, including an initial pneumonectomy prior to monocrotaline injection. Exposure to chronic hypoxia also induces pulmonary hypertension in many animals as well as humans, and essentially recapitulated secondary pulmonary hypertension due to chronic lung disease(38). Chronic hypoxia exposure is commonly used to induce pulmonary hypertension in both rats and mice. The fawn hooded rat model, in which the rats have a mitochondrial disorder and develop, among other things, spontaneous pulmonary hypertension at normal oxygen levels(14). Other models exist that recapitulate more specific forms of pulmonary hypertension, such as the neonatal lamb model of persistent pulmonary hypertension of the newborn, or the rat biliary ligation model of portopulmonary hypertension. Of all these models, the model that most closely recapitulates human pulmonary arterial hypertension is arguably the monocrotaline model.

Hypothesis & Specific aims

The central hypothesis of this work is that, with either pharmacologic or gene therapy, targeting the abnormal growth and proliferation of cells in the resistance pulmonary arterial bed would prevent or reverse the remodeling of PAH, and induce corresponding improvements in hemodynamics, morbidity, and mortality.

1) To show that the orally available metabolic modulator dichloroacetate (DCA), which enhances oxidative phosphorylation by inhibiting the mitochondrial pyruvate dehydrogenase kinase would prevent and reverse monocrotaline-induced PAH (MCT-PAH) in rats by inducing mitochondria dependent apoptosis and reversing the downregulation of Kv1.5 in the media of resistance PAs.

2) To show that survivin is expressed in remodeled resistance PAs, but not normal PAs, from PAH patients and rats with MCT-PAH, and to show that inhaled adenoviral gene therapy with survivin-M would reverse MCT-PAH by induction of mitochondria-dependent apoptosis in PASMCs.

3) To show that combination therapy with high dose rapamycin 2.5 mg/kg/day and atorvastatin 10mg/kg/day would have synergistic beneficial effects on the pulmonary vasculature, and would reverse established MCT PAH in rats.

4) To show that overexpression of BMPR2 in the pulmonary vascular tree using an adenoviral gene therapy approach would ameliorate or reverse established MCT-PAH in rats.

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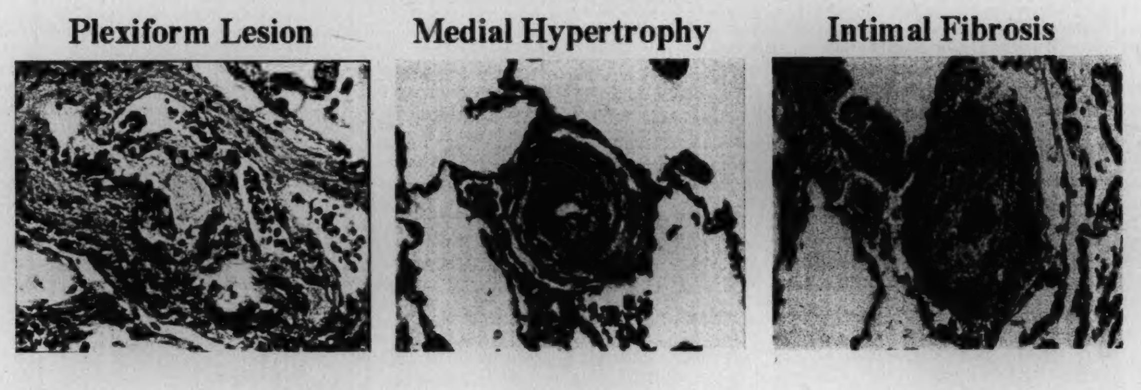
Figure 1 - 1

Histologic Changes of PAH



Plexiform Lesion

Medial Hypertrophy

Intimal Fibrosis

Dichloroacetate Prevents and Reverses Pulmonary Hypertension by Inducing Pulmonary Artery Smooth Muscle Cell Apoptosis

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Citation for this article: *Circ. Res.* 2004;95;830-840.

Abstract

The pulmonary arteries (PA) in pulmonary arterial hypertension (PAH) are constricted and remodeled; They have suppressed apoptosis, partly attributable to suppression of the bone morphogenetic protein axis and selective downregulation of PA smooth muscle cell (PASMC) voltage-gated K+ channels, including Kv1.5. The Kv downregulation-induced increase in [K+]i, tonically inhibits caspases, further suppressing apoptosis. Mitochondria control apoptosis and produce activated oxygen species like H_2O_2, which regulate vascular tone by activating K+ channels, but their role in PAH is unknown. We show that dichloroacetate (DCA), a metabolic modulator that increases mitochondrial oxidative phosphorylation, prevents and reverses established monocrotaline-induced PAH (MCT-PAH), significantly improving mortality. Compared with MCT-PAH, DCA-treated rats (80 mg/kg per day in drinking water on day 14 after MCT, studied on day 21) have decreased pulmonary, but not systemic, vascular resistance (63% decrease, $P<0.002$), PA medial thickness (28% decrease, $P<0.0001$), and right ventricular hypertrophy (34% decrease, $P<0.001$). DCA is similarly effective when given at day 1 or day 21 after MCT (studied day 28) but has no effect on normal rats. DCA depolarizes MCT-PAH PASMC mitochondria and causes release of H_2O_2 and cytochrome c, inducing a 10-fold increase in apoptosis within the PA media (TUNEL and caspase 3 activity) and decreasing proliferation (proliferating-cell nuclear antigen and BrdU assays). Immunoblots, immunohistochemistry, laser-captured microdissection-quantitative reverse-transcription polymerase chain reaction and patch-clamping show that DCA reverses the Kv1.5 downregulation in resistance PAs. In summary, DCA

reverses PA remodeling by increasing the mitochondria-dependent apoptosis/proliferation ratio and upregulating Kv1.5 in the media. We identify mitochondria dependent apoptosis as a potential target for therapy and DCA as an effective and selective treatment for PAH.

Introduction

Pulmonary arterial hypertension (PAH) is defined by an elevated pulmonary vascular resistance (PVR), which leads to right heart failure and premature death. The cause remains unknown and available treatments are limited, expensive, and often associated with significant side effects[1, 2]. The pulmonary arteries (PAs) are affected by varying degrees of vasoconstriction and vascular remodeling, including cellular proliferation in both the intima and media and distal PA muscularization[1, 2].

Vascular medial remodeling results from an imbalance between smooth muscle cell (SMC) proliferation and apoptosis, favoring proliferation. Gene microarray studies show that lungs from patients with PAH have a decrease in the proapoptotic/antiapoptotic gene expression ratio[3]. Furthermore, several loss-of-function germline or acquired mutations have been described in receptors of the transforming growth factor-β superfamily, such as bone morphogenetic protein receptor-2 (BMPR-2), in patients with primary PAH[2]. Activation of the transforming growth factor-β/BMPR2 axis suppresses proliferation and activates apoptosis in normal PA smooth muscle cell (PASMC)[4] but not in PASMC from patients with PAH[5]. This resistance to apoptosis is further enhanced by the selective downregulation of Kv channels that has been shown in human[3,6] and animal models of PAH[7] Intracellular K+ levels, which increase when K+ channels are inhibited or downregulated, exhibit a tonic inhibition of caspases in many cell types, including PASMC[8]. In addition to a suppression in apoptosis, Kv channel downregulation leads to PASMC depolarization, opening of the voltage-gated Ca++ channels, and increased intracellular Ca++; in turn, this causes vasoconstriction and increased PASMC proliferation[9].

The role of mitochondria in pulmonary vascular biology and PAH is unknown. However, mitochondria are potentially important because they regulate both apoptosis (by the release of proapoptotic factors, including the caspase activator cytochrome c[10]) and vascular tone (by the production of activated oxygen species [AOS]). AOS can "leak" to the cytoplasm and affect redox-sensitive second messenger systems and membrane K+ channels. For example, superoxide is produced in the proximal electron transport chain (ETC) and, in the presence of the mitochondria-based manganese superoxide dismutase (MnSOD), it is dismutated to H_2O_2, a well-characterized K+ channel opener and vasodilator[11, 12]. Therefore, vascular mitochondria might be important targets for the treatment of vascular disease.

We show that the orally available metabolic modulator dichloroacetate (DCA), which enhances oxidative phosphorylation by inhibiting the mitochondrial pyruvate dehydrogenase kinase and has been used extensively in humans for mitochondrial diseases and lactic acidosis[13], prevents and reverses monocrotaline-induced PAH (MCT-PAH) in rats. DCA inhibits MCT-PAH by inducing mitochondria dependent apoptosis and reversing the downregulation of Kv1.5 in the media of resistance PAs.

Materials and Methods

Experimental Protocols. Experiments were in accordance with the University of Alberta Animal Policy and Welfare Committee. MCT-PAH was induced by injection of MCT 60 mg/kg subcutaneously on day 1. Treated rats (MCT DCA) were fed with DCA (0.75 g/L, pH 7.0; Sigma-Aldrich) in drinking water, with an average ingestion of 80 mg/kg per day[7], unless stated otherwise (see dose-response experiments). A total of 20 normal control rats (15 with no treatment and 5 with DCA treatment) were used. In the protocols listed, all rats were hemodynamically studied and tissues obtained for additional studies.

DCA was started on day 1. Treated (n=20) and nontreated MCT-PAH (n=10) rats were studied on day 21. For early reversal, DCA was started on day 14. Treated (n=10) and nontreated MCT-PAH (n=10) rats were studied on day 21. For late reversal, DCA was started on day 21. Treated (n=5) and nontreated MCT-PAH (n=9) rats were studied on day 28.

In additional time-course experiments, MCT-PAH rats were studied on days 0, 7, 14, 21, 28 (n=3 for each) and in parallel with additional MCT-DCA day 14 (n=5) and MCT-DCA day 21 (n=5) rats. Three additional MCT-PAH rats were followed-up from day 0 to day 28 with implanted telemetry catheters.

For dose-reponse studies MCT-PAH rats (n=10) were compared with rats treated with the following doses of DCA (g/L): 0.075 (n=3), 0.75 (n=4), and 7.5 (n=3), using the early reversal protocol. The rats included in all the protocols (MCT-PAH, n=51; MCT-DCA, n=55) were prospectively followed-up for survival analysis. Spontaneous deaths were counted as events. Rats undergoing planned hemodynamic evaluation and euthanization were censored. Time to event data were plotted using the Kaplan-Meier method, with differences evaluated using the log rank test.

Echocardiology. We used a SONOS 5500 machine with 15-Mhz and 12-Mhz probes (Philips Medical Systems). Right ventricular (RV) free-wall thickness, measured by M mode, and PA Doppler signals were measured in parasternal short axis[14].

Hemodynamics. Rats anesthetized with pentobarbital (50 mg/kg intraperitoneally) were orotracheally intubated, ventilated (FiO2 0.4), and left heart (left ventricular end-diastolic pressure), carotid artery (arterial pressure), and PA catheterization were performed as described[7, 15] using 1.4-French Millar catheters (Millar Instruments). Cardiac index was measured using the Fick and thermodilution methods as previously described[7, 15]: Pulmonary vascular resistance index (PVRi) was calculated as (mean PA - left ventricular end diastolic pressure)/cardiac index.

Morphometry, Immunoblotting, Immunohistochemistry, and Quantitative RT-PCR. For morphometric analysis, RV hypertrophy was measured as RV/(left ventricular septum) weight and PA remodeling was measured as percent medial thickness[7,15]. Immunoblotting was performed on pooled PA samples (each band has PAs pooled from 3 rats/group; 25 μg protein/pooled sample), as described[7,11,15]. Paraffin-embedded lungs were sectioned and stained with antibodies to either Kv1.5 or Kv2.1 and counterstained with eosin, as described[15,16]. Laser capture microdissection and quantitative RT-PCR were performed as previously described[15,17,18].

Electrophysiology. Fresh PASMC were isolated from fourth to sixth division PAs and studied with whole-cell patch-clamping, as described[7,11,15].

Confocal Microscopy. Imaging was performed using a Zeiss LSM 510 confocal microscope as described[7,15,17]. Apoptag apoptosis detection kit (TUNEL stain; Serologicals, Norcross, Ga) and the proliferating cell nuclear antigen (PCNA) antibody (DAKO, Carpinteria, Calif) were used as per manufacturer's instructions on paraffin-embedded tissue sections after antigen retrieval (microwave). Counterstain with 4,6 -diamidino-2-phenylindole dihydrochloride (DAPI, 300 nM; Molecular Probes) was performed for 10 minutes at 20°C and washed with phosphate-buffered saline. Apoalert Annexin V kit (Clontech, Palo Alto, Calif) and cytochrome c antibody (Pharmingen, San Diego, Calif) were used as per manufacturer's instructions on cells exposed to either propidium iodide (500 nM; Molecular Probes, Eugene, Ore) or DAPI. BrdU was injected (100 mg/kg intraperitoneally) 4 hours before euthanization, and lungs were formalin-fixed and paraffin-embedded. Staining of sections was performed as per manufacturer's instructions (Hoffman-La Roche).

Dichlorofluorescein Assay. For dichlorofluorescein assay, PAs were incubated in Krebs buffer supplemented with 10 μmol/L dichlorofluorescein (Molecular Probes), and H_2O_2 production was measured as described[11]. In brief, production of H2O2 by isolated RA or PAs was measured by oxidation of 10μM 2',7'-dichlorofluorescin diacetate (DCF) (Molecular Probes, Eugene OR) in Krebs buffer at 37°C over a sixty minute interval.

Aqueous sodium cyanide was used at 1μM concentrations, myxothiazol 100ng/ml (in ethanol) and rotenone at 10μM (in DMSO) (Sigma Aldrich, Oakville ON.) Drugs were tested under normoxia. Fluorescence resulting from oxidation of DCF was detected using a spectrofluorometer, (Spectra Max Gemini XS, Molecular Probes, Eugene OR) with excitation at 495nm and emission at 530nm. Background was corrected for by running controls containing the appropriate drug. Relative Fluoresence (RF) was calculated as follows (FU=fluorescence units):

Sample fluorescence (S)= sample FU60min – sample FU0min

Control fluorescence (C) control FU60min-control FU0min

RF = S - C/Tissue wt (mg)

Statistics. Values are expressed as the mean±SEM. Intergroup differences were assessed by Kruskal-Wallis or 1-way ANOVA as appropriate with post hoc analysis using Fisher exact test (Statview 4.02; SAS Institute, Cary, NC).

Results

DCA Therapy Improves Survival Without Evidence of Toxicity. We studied the rats within 4 weeks after MCT injection to prevent the animals from experiencing the severe heart failure and morbidity associated with the rapid worsening of PAH beyond 4 weeks[19,20] DCA significantly improves survival in MCT-PAH and the positive effect on survival is present 1 week after initiation of treatment (Figure 1A). Furthermore, the DCA-treated rats showed no evidence of liver, renal, or blood toxicity.

DCA Normalizes the PVRi in PAH Without Affecting Systemic Hemodynamics. DCA-treated rats had significantly lower PVRi compared with nontreated MCT-PAH rats (Figure 1B, 1C), whereas arterial pressure (Figure 1C) and left ventricular end-diastolic pressure were not different. The PVRi of the DCA-treated rats in both the prevention and reversal protocols was normalized, ie, it was not statistically different from normal controls. The significant hemodynamic improvement in the DCA-treated rats was also confirmed echocardiographically. The shift of the interventricular septum toward the left ventricle, an index of severe RV pressure overload, was normalized in all DCA-treated rats (Figure 1C).

Another clinically useful physiologic index of PAH is the PA acceleration time (PAAT), ie, the time from the beginning to the peak of the velocity envelop during pulsed Doppler interrogation of the pulmonary valve[14] (Figure 1D). We show a strong correlation between PAAT and PVRi in rats simultaneously studied with ECHO and catheterization (the higher the PA pressure, the shorter the PAAT) (Figure 1D). PAAT was significantly decreased in the MCT rats versus controls and was increased in rats that received DCA both on day 14 and day 21 (Figure 1D).

Most of our experiments were performed with 0.75g/L, because at this dose DCA has been used safely and effectively in humans with metabolic disorders[13]. To determine whether there is a dose response, in some rats we also used a lower and a higher dose (Figure 1D). We showed that the low dose 0.075g/L is probably a threshold dose, having minimal effects on hemodynamics. The high 7.5-g/L dose had only minimal additional effects compared with 0.75 g/L. However, at the high 7.5-g/L dose, the water tends to have a moderate odor and bitterness and, although not significant, the rats consumed less water plus DCA.

As predicted, DCA also caused reversal of RV hypertrophy (RVH) measured both echocardiographically (Figure 1D) and by RV/left ventricular septum weight ratio. In keeping with the PAAT dose response data, the effects of the low dose DCA on RVH were minimal, whereas the two higher doses caused essentially a complete reversal of RVH (Figure 1D).

DCA Reverses PA Remodeling by Inducing Apoptosis and Suppressing Proliferation in the Media. As predicted by the hemodynamic data, DCA prevented and reversed the percent medial hypertrophy in MCT-PAH (Figure 2A). The media of the remodeled MCT-PAH PAs showed evidence of enhanced cell proliferation, with increased BrdU uptake (Figure 2B) and PCNA expression (Figure 2C), whereas in DCA-treated animals both BrdU uptake and PCNA expression were completely suppressed. Whereas the MCT-PAH PAs had no evidence of apoptosis (measured by TUNEL and propidium iodide-stained nuclear morphology), DCA-treated rats had a significant increase (~10-fold) in apoptosis (Figure 2C). In some MCT-PAH PAs, extensive PCNA expression and no apoptosis were seen in the absence of significant medial hypertrophy, suggesting that proliferation likely precedes, eventually leading to, PA remodeling. Essentially all the PCNA, BrdU, and TUNEL-positive nuclei were seen in the PA media and adventitia and not in the endothelium. Furthermore, DCA treatment caused activation of an important effector of apoptosis, caspase 3, in the PAs (Figure 3A).

DCA-Induced Apoptosis Is Mitochondria-Dependent, Accompanied by Increased H_2O_2 Production, and Occurs Early. Isolated PASMC from MCT-PAH rats had a significant increase in mitochondria membrane potential (ΔΨm) versus control rats, and this increase was present even at 7 days after MCT injection, a time when the hemodynamic changes of PAH are not yet established. DCA treatment (early reversal protocol) decreased the ΔΨm back to normal levels (Figure 3B).

Because AOS production increases when ΔΨm depolarizes, and because the PASMC mitochondria are enriched in manganese superoxide dismutase (at least compared with systemic vascular SMC[11]), we studied H_2O_2 production from freshly isolated PAs. Compared with controls, PAs isolated from MCT-PAH rats make less H_2O_2. The production of H_2O_2 increases to normal levels in the DCA-treated rats (Figure 3C). To determine whether DCA (100 μmol/L) had any acute effects, we incubated the MCT-PAH PAs for 1 hour with DCA, and we showed that DCA increased the H_2O_2 production to the level of the rats chronically treated with DCA. We also showed that this acute increase was prevented by rotenone (5 μmol/L, an inhibitor of the complex I of the mitochondrial ETC) (Figure 3C).

To determine whether DCA would have the same effects on mitochondria in vitro and in another model, we used chronically hypoxic PASMC (CH-PASMC) in primary culture. This model is relevant to PAH because chronic hypoxia is the most common cause of clinical PAH. Furthermore, we have recently shown that DCA reversed rat chronic hypoxia-induced PAH, but in that article we did not explore the effects of DCA on mitochondria and apoptosis pathways[7]. DCA treatment (100 μmol/L) caused CH-PASMC apoptosis measured by TUNEL and nuclear morphology (Figure 4A), which occurred as early as 6 hours, as shown by annexin straining (Figure 4B). DCA treatment caused leakage of the caspase activator cytochrome c from mitochondria (Figure 4C). DCA depolarized CH-PASMC mitochondria (Figure 4D). We also measured H_2O_2 production in freshly isolated PAs placed in hypoxic tissue culture for 8 hours. Exposure to DCA (100 μmol/L) increased and normalized H_2O_2 production in hypoxic PAs. DCA did not affect H_2O_2 production in normoxic PAs (not shown).

DCA Does Not Affect Normal PASMC and PAs. DCA does not affect the $\Delta\Psi m$ of freshly isolated PASMC from healthy rats and normal PASMC in primary culture (Figure 5A). Importantly, DCA does not induce apoptosis in normal PASMC (Figure 5B) and does not affect hemodynamics in healthy rats treated with DCA (Figure 5C) for the same duration and dose as the MCT-PAH rats.

DCA Treatment Reverses the Downregulation of PASMC Kv1.5 in MCT-PAH. We found a significant decrease in K+ current in freshly isolated PASMC from the MCT-PAH rats, compared with the controls (Figure 6A,B), which was improved by the prevention and reversal DCA treatment protocols (Figure 6A, 6C). Most of the suppressed K+ current in the MCT-PAH PASMC is 4-aminopyridine–sensitive (ie, Kv current), with a much smaller component being iberiotoxin-sensitive (ie, large conductance calcium-activated, BKCa current). The decrease in the Kv was both prevented and reversed by DCA (Figure 6D).

Although many K+ channels are active in the PAs, Kv channels, and specifically Kv1.5 and Kv2.1, are the ones controlling PASMC membrane potential[21] and are the ones primarily expressed in small resistance PAs[18, 22]. To molecularly identify the Kv current modulated by DCA treatment, we measured expression of protein and mRNA for Kv.1.5 and Kv2.1. The protein levels of Kv1.5 and Kv2.1 channels in MCT-PAH PAs are decreased, compared with the controls (Figure 6E, 6F). DCA treatment almost completely reverses the downregulation of Kv1.5 but not Kv2.1 (Figure 6E, 6F), in agreement with our electrophysiology data.

Both Kv1.5 and Kv2.1 mRNA levels were significantly decreased in laser-capture microdissected resistance (25 to 50 μm) PAs of the MCT-PAH compared with the normal control rats (Figure 6G). DCA treatment partially reversed the downregulation of Kv1.5 ($P<0.05$) but not Kv2.1 (although there was a trend, $P=0.06$).

Changes in K+ Channels and Apoptosis Parallel the Hemodynamic Effects of MCT and DCA Treatment. To characterize the timing of the development of MCT-PAH and the temporal relation between the observed hemodynamic and molecular effects of MCT and DCA treatment, we performed additional time-course experiments. We used telemetry catheters and ECHO to prospectively measure hemodynamics, so that rats would serve as their own controls. At day 14, the time that our early reversal protocol begun, PAH is mild but clearly established, as shown by direct PA measurement with telemetry (Figure 7A) and PAAT (Figure 7B). That the PA pressure increase is significant is also shown by the fact that RVH is present (Figure 7B). We performed quantitative reverse-transcription polymerase chain reaction in whole lungs from these animals and we show that Kv1.5 and Kv2.1 downregulation precedes the development of PAH (Figure 7C). Kv2.1 is maximally suppressed as early as day 7 after MCT and Kv1.5 is maximally suppressed at day 14; expression of both channels decreases minimally beyond that point, whereas the maximal effects in hemodynamics occur between day 14 and day 21. Both MCT and DCA had minimal and no significant effects on Kir2.1. In agreement with our protein expression and laser capture microdissection–quantitative reverse-transcription polymerase chain reaction data (Figure 6), these whole-lung data show that DCA treatment upregulates Kv1.5 but not Kv2.1.

We found a trend for a decrease in apoptosis during the first 2 weeks after MCT (which did not reach statistical significance). Apoptosis shows a tight temporal relation with both the hemodynamic and Kv channel effects of DCA because there is a 12-fold (~600% increase, similar to our data in Figure 2C) in apoptosis from day 14 to day 21 (Figure 7C). The TUNEL-positive cells were all in the media and adventitia, as in Figure 2.

Discussion

We show that DCA can both prevent and reverse established pulmonary vascular remodeling in a common model of PAH and thus improve hemodynamics, RVH, and survival. We show that DCA increases the apoptosis/proliferation ratio in the media of remodeled PAs, without affecting healthy tissues or systemic vessels. DCA depolarizes PASMC mitochondria, thus initiating mitochondria-dependent apoptosis. DCA also reverses the downregulation of Kv current that occurs in MCT-PAH, and by this mechanism it further enhances apoptosis and suppresses proliferation (Figure 8). Our work shows for the first time to our knowledge that mitochondria dependent apoptosis in the vascular wall is important for vascular remodeling and that the mitochondria and K+ channel axis can be targeted therapeutically in PAH. Because DCA is selective for the pulmonary circulation, has a very good safety profile in PAH rats and humans[13], and is orally available, it is a very attractive potential therapy for human PAH, a disease in which effective, simple to deliver, and nontoxic therapies are urgently needed.

The degree of DCA-induced apoptosis in the PA media is in agreement with a recent study showing that the regression of PA vascular remodeling caused by elastase inhibitors is associated with induction of apoptosis (measured by TUNEL) and a decrease in cell proliferation (measured by PCNA expression) in the media of the PAs[20]. Similarly, Nishimura et al recently showed that simvastatin caused regression of vascular remodeling by inducing vascular PASMC apoptosis[23]. These two studies and our results confirm the hypothesis that PAH might be an apoptosis resistance state and that pharmacological enhancement of apoptosis would be therapeutically beneficial. In contrast, Zhao et al showed that apoptosis is increased in MCT-PAH and that suppressing apoptosis with angiopoietin gene transfer prevented PAH[19]. These data are not necessarily conflicting, because Zhao et al studied endothelial cell apoptosis and intervened early, when apoptosis in the endothelial cells in capillaries and very small PAs might indeed be increased, perhaps as a direct result of MCT, a known endothelial toxin. We focus on PASMC apoptosis and in our reversal protocol, we intervene late in already remodeled PAs. Intervening in established PAH is more relevant clinically, because patients typically present late. We did not find any evidence of increased apoptosis in the endothelial cells (at least on day 7 after MCT; Figure 7C), although this observation is limited by the fact that we did not use an endothelial marker to look for very small arteries and capillaries. More studies are needed to clarify the intriguing hypothesis that apoptotic mechanisms might show diversity within the vascular wall segments and might also vary with the age of the remodeling process.

Our findings that DCA depolarizes PASMC mitochondria early and causes release of mitochondrial cytochrome c (Figure 4) provide a potential mechanism for the apoptosis that we observed in vivo. The initiation of mitochondria dependent apoptosis can be further potentiated downstream by the decrease of intracellular K+ that follows the DCA-induced activation of Kv channels[7] and the upregulation of Kv1.5 expression. Kv1.5 might be a key player in PASMC and may regulate apoptosis, much like Kv2.1 modulates apoptosis in neurons[24]. Both the increase in cytochrome c and the decrease in intracellular K+ explain the activation of caspase 3 that we observed in the PA (Figure 3A).

In human PAH, receptor-mediated apoptosis is suppressed, at least in part, because the BMPR-2 axis is suppressed. Cell culture studies indicate that BMP-2 normally causes K+ channel-dependent apoptosis of SMCs, which may not occur in cells with the BMPR-2 mutation[4]. In other words, K+ channels modulate apoptosis distal to both receptor-mediated and mitochondria-mediated apoptosis. DCA can directly increase apoptosis and activate K+ channels. Although both the increase in cytochrome c and H_2O_2 explain the activation of Kv channels, the mechanism by which DCA increases Kv1.5 mRNA expression, and whether this is related to its effects on mitochondria, remains unknown. However, this potential synergy of mechanisms (ie, mitochondria and Kv channels) might explain why DCA is so effective in PAH, normalizing PVRi and RVH (Figure 1). For example, we recently showed that simply replenishing Kv1.5 with gene therapy improved rat CH-PAH, but the effects on hemodynamics and RVH were only modest [15].

How would DCA cause the opening of the mitochondria transition pore (MTP) and mitochondrial depolarization? MTP is redox-sensitive and an increase in electron transport chain production of AOS can cause the opening of the MTP and mitochondrial depolarization[10]. DCA enhances mitochondrial oxidative phosphorylation by increasing the levels of intramitochondrial pyruvate and acetyl-CoA levels, which follows pyruvate dehydrogenase kinase inhibition[13]. The increase in the acetyl-CoA that enters the Krebs cycle causes an increase in the NADH/NAD ratio in the mitochondrial matrix, which in turn increases the AOS produced in the complex I of the ETC[25]. PASMC mitochondria have very high levels of MnSOD, compared with systemic SMC mitochondria, and thus superoxide is preferentially dismutated to H_2O_2 [11], a well-known stimulant of mitochondrial-dependent apoptosis and opener of the MTP[26–28]. DCA acutely increases the production of H_2O_2 in PAs (Figure 3C and Figure III), and this increase is occurring in complex I of the ETC, because it is inhibited by rotenone (Figure 3C).

Although the doses that we used in vitro and in vivo are in agreement with the doses required to inhibit PDH kinase (and thus activate PDH), we have previously shown that at 10 μmol/L (a dose thought to be too small to inhibit PDH kinase) DCA activates expressed Kv channels in vitro and that this activation is tyrosine kinase-dependent[7]. It is possible that DCA could have 2 mechanisms, involving tyrosine kinase at very low doses and PDH kinase at higher doses, although we cannot rule out PDH kinase isozymes in the resistance PASMC with increased sensitivity to DCA. A limitation of this study is that precise metabolic studies measuring glucose and fatty acid oxidation were not performed.

Our proposed mechanism for the effects of DCA on mitochondria-derived H_2O_2, K+ channel function and apoptosis (Figure 8) resembles a recently proposed mechanism for HERG (a K+ channel important in the regulation of myocardial repolarization) on regulating apoptosis of tumor cells[29]. H_2O_2-induced apoptosis in tumor cell lines is suppressed by pharmacological or molecular inhibition of HERG[29]. A similar mechanism might occur in the "K+ channel-deficient" PAH, the primary form of which has been proposed to be a form of vascular neoplasia[30]. We recently showed that the PASMC have relatively depolarized mitochondria, more MnSOD, and tonically produce more H_2O_2 compared with systemic vascular SMC mitochondria[11]. In PAH, the "protective" effects of H_2O_2 in both tone and apoptosis might be inhibited because of the deficiency of Kv channels, contributing to PASMC contraction and proliferation.

DCA has no effects on normal PASMC (Figure 5), in agreement with our previous report that DCA increases Kv current in hypoxic, but not normoxic, CHO cells expressing Kv2.1.7 This is also in agreement with the fact that DCA activates K+ current in myocardial cells from an infarcted area but not from healthy myocardium[31]. This is important clinically because DCA might "target" mitochondria only in abnormal or proliferating PASMC, minimizing possible toxicity to healthy cells and tissues.

Another clinically attractive property of DCA is that its effects are specific to the pulmonary circulation, because it does not alter systemic hemodynamics (Figure 1)[7]. Often the treatment of PAH patients is limited by the systemic hemodynamic effects of therapies, causing hypotension. The mechanism for the selectivity of DCA to the pulmonary circulation is unknown, but the differences of the PASMC mitochondria compared with systemic SMC mitochondria[11] raise the possibility that PASMC mitochondria are more sensitive to the effects of pyruvate dehydrogenase kinase inhibition.

The present study and our recent studies[7] suggest that DCA is an attractive treatment for human PAH and provide the rationale for initiation of a clinical trial in humans with PAH. Our study also suggests that the interplay of mitochondria, membrane Kv channels, and apoptosis might provide novel pharmacological targets for the treatment of vascular disease.

Acknowledgments

E.D.M. receives support from the Canadian Institutes for Health Research, Alberta Heritage Foundation for Medical Research, Heart and Stroke Foundation, Canadian Foundation for Innovation and the Alberta Cardiovascular and Stroke Research Centre (ABACUS).

M.S.M. receives fellowship support from Bristol-Myers Squibb.

Figure Legends

Figure 2-1 - DCA improves hemodynamics in MCT-PAH. A, Kaplan-Meyer curve showing a significant survival benefit in the DCA-treated rats compared with the untreated MCT-PAH rats (n=55 and 51, respectively). Representative high-fidelity PA pressure tracings (B) and mean PVRi and systemic blood pressure data (C). Representative echocardiograms demonstrating septal shift in MCT-PAH, reversed by DCA treatment. D, Regression analysis shows that PAAT predicts invasively measured PVRi. ECHO data on the early (3 different doses) and late reversal protocols show that DCA reverses the PAAT shortening and RVH caused by MCT (*P<0.01 versus MCT).

Figure 2-2 - DCA reverses PA remodeling by increasing the apoptosis/proliferation ratio in the media. A, The percent medial thickness of resistance PAs is reduced by DCA-treatment (*P<0.05 versus MCT, n=60 PAs/group; 2 to 3 slides/rat, 3 rats/group). B and C, Representative images of MCT and MCT-DCA rat resistance PAs. In the MCT PAs, there is BrdU uptake and heavy expression of PCNA in the media, whereas there are no TUNEL-positive nuclei. In contrast, PAs from the DCA-treated rats have suppressed BrdU uptake and PCNA expression and a significant increase in the TUNEL-positive cells in the media. *P<0.001 versus MCT, n=100 PAs/group; 2 to 3 slides/rat; 5 rats/group. L indicates Lumen.

Figure 2-3 - DCA increases H2O2 production, depolarizes mitochondria, and activates caspase 3 in MCT-PAH PAs in vivo. A, Caspase-3 in pooled PAs from MCT-DCA rats

is cleaved (activated), compared with untreated MCT and control rats. B, DCA treatment normalizes the ΔΨm in PASMC, compared with PASMC from untreated MCT PAs (n=20/group, cells from 3 rats/group). C, Chronic treatment with DCA normalizes the suppression of H_2O_2 production seen in MCT-PAH. Acute treatment with DCA increases H_2O_2 production and this is prevented by rotenone. *$P<0.05$ versus MCT, n=3/group.

Figure 2-4 - DCA induces mitochondria-dependent apoptosis in vitro. A, Hypoxic PASMCs in culture show low rates of apoptosis measured by TUNEL. Treatment with DCA100 μmol/L for 24 hours increases apoptosis. *$P<0.05$ versus hypoxia+vehicle, n=20 plates/group, 4 random fields/plate. Cells are identified as PASMC by their smooth muscle actin positivity (triple-stained with DAPI-blue, mitotracker-red and SMA-green). TUNEL-positive PASMC nuclei were shrunken and pyknotic, consistent with late stages of apoptosis. B, DCA (100 μmol/L for 6 hours) induces expression of annexin on the plasma membrane of hypoxic PASMCs. Two necrotic cells are seen in the image of vehicle-treated cells (positive for both annexin and propidium iodide [PI]). The cells in the DCA-treatment panels are apoptotic because they are annexin-positive and PI-negative. C, Hypoxic vehicle-treated PASMCs do not show release of cytochrome c from mitochondria into the cytoplasm (discrete, punctate staining pattern). DCA treatment (100 μmol/L for 6 hours) causes release of cytochrome c (diffuse staining of the cytoplasm). D, Vehicle-treated hypoxic PASMC mitochondria are hyperpolarized, whereas PASMC mitochondria from DCA-treated rats are depolarized, as shown by the reduced TMRM fluorescence (n=20/group).

Figure 2-5 - DCA has no effects on normal PASMC. DCA does not affect ΔΨm (n=20) (A) and apoptosis in normal PASMC (n=10plates/group, 4 random fields/group) (B). C, DCA does not alter PVRi in normal rats (n=5/group).

Figure 2-6 - DCA reverses the Kv1.5 downregulation. A to D, PASMC from MCT rats (n=10) have markedly decreased K+ current density, which is both prevented (n=9) and reversed (n=5) by DCA (*P<0.05 versus control). Representative traces and current density mean data are shown. The percent increase in the current is almost identical whether DCA is given on day 1 or on day 14. DCA normalizes the 4-AP–sensitive current, which is significantly suppressed in untreated MCT rats. E and F, Immunohistochemistry (left: control; middle: MCT-PAH; right: DCA-treated; Kv1.5 in brown) and immunoblots show Kv1.5 and Kv2.1 protein expression is decreased in the PAs of MCT rats, but only Kv1.5 is augmented by DCA. (*P<0.05 versus MCT). G, Quantitative reverse-transcription polymerase chain reaction of laser-captured resistance PAs demonstrates that Kv1.5 and Kv2.1 mRNA is decreased in MCT, but only Kv1.5 is partially restored by DCA (*P<0.05 versus MCT, n=15, 3 rats/group).

Figure 2-7 - The K+ channel modulation and apoptosis closely parallel hemodynamics in MCT-PAH. A, Continuous recording of mean PA pressure in a freely moving untreated MCT-PAH rat with implanted telemetry catheter (representative of three). ECHO data (B), whole lung K+ channel expression (quantitative reverse-transcription polymerase

chain reaction), and apoptosis (percent TUNEL-positive cells) (C) in the time course experiments.

Figure 2-8 - A proposed mechanism for the effects of DCA on pulmonary vascular remodeling.

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Figure 2-1

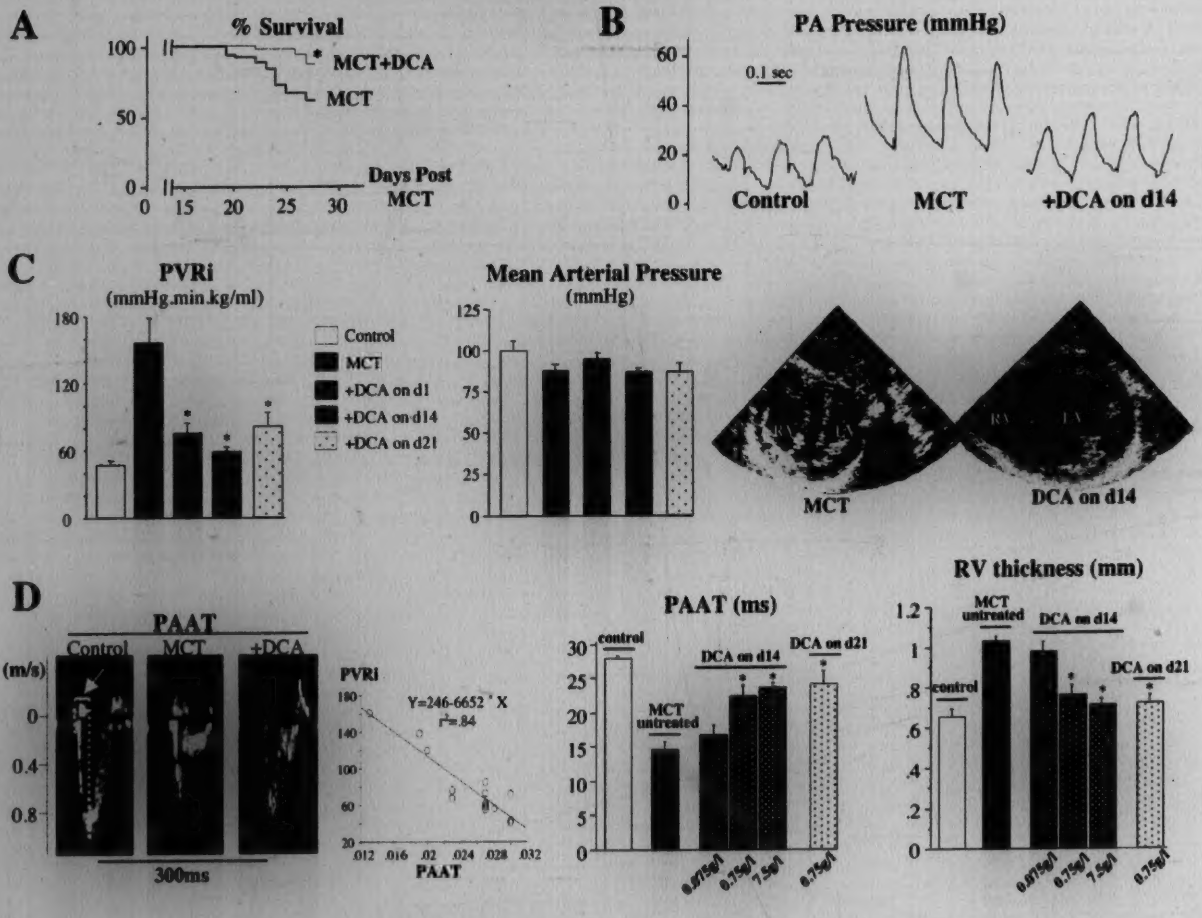

A
% Survival
100
50
0
0
15
20
25
30
Days Post MCT
MCT+DCA
*
MCT
B
PA Pressure (mmHg)
60
40
20
0
0.1 sec
Control
MCT
+DCA on d14
C
PVRi
(mmHg.min.kg/ml)
180
120
60
0
*
*
*
Control
MCT
+DCA on d1
+DCA on d14
+DCA on d21
Mean Arterial Pressure
(mmHg)
125
100
75
50
25
0
RV
LV
MCT
DCA on d14
D
PAAT
Control
MCT
+DCA
(m/s)
0
0.4
0.8
300ms
PVRi
180
140
100
60
20
Y=246-6652 * X
r²=.84
.012
.016
.02
.024
.028
.032
PAAT
PAAT (ms)
30
25
20
15
10
5
0
control
MCT untreated
DCA on d14
DCA on d21
*
*
*
0.075g/l
0.75g/l
7.5g/l
0.75g/l
RV thickness (mm)
1.2
1
.8
.6
.4
.2
0
control
MCT untreated
DCA on d14
DCA on d21
*
*
*
0.075g/l
0.75g/l
7.5g/l
0.75g/l

Figure 2-2

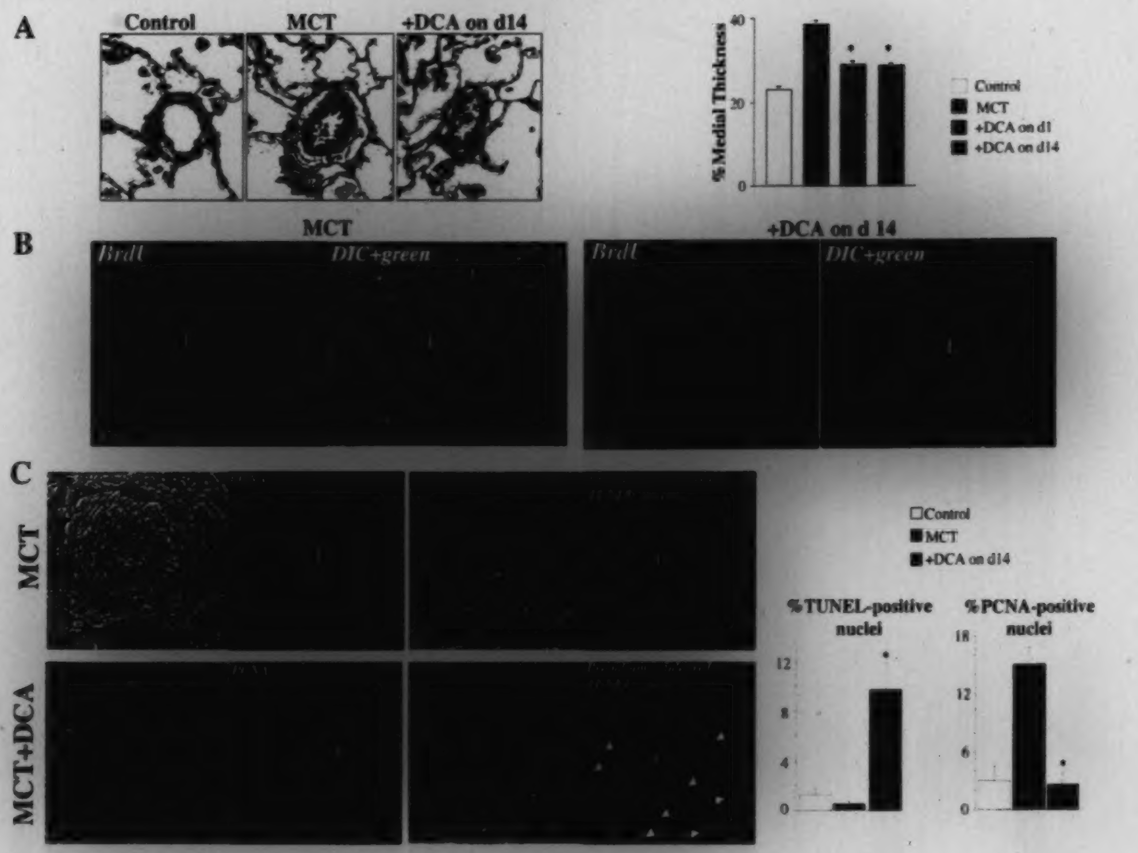

A
Control
MCT
+DCA on d14
% Medial Thickness
40
20
0
Control
MCT
+DCA on d1
+DCA on d14
B
MCT
+DCA on d 14
BrdU
DIC+green
BrdU
DIC+green
C
MCT
MCT+DCA
Control
MCT
+DCA on d14
% TUNEL-positive nuclei
12
8
4
0
% PCNA-positive nuclei
18
12
6
0

Figure 2-3

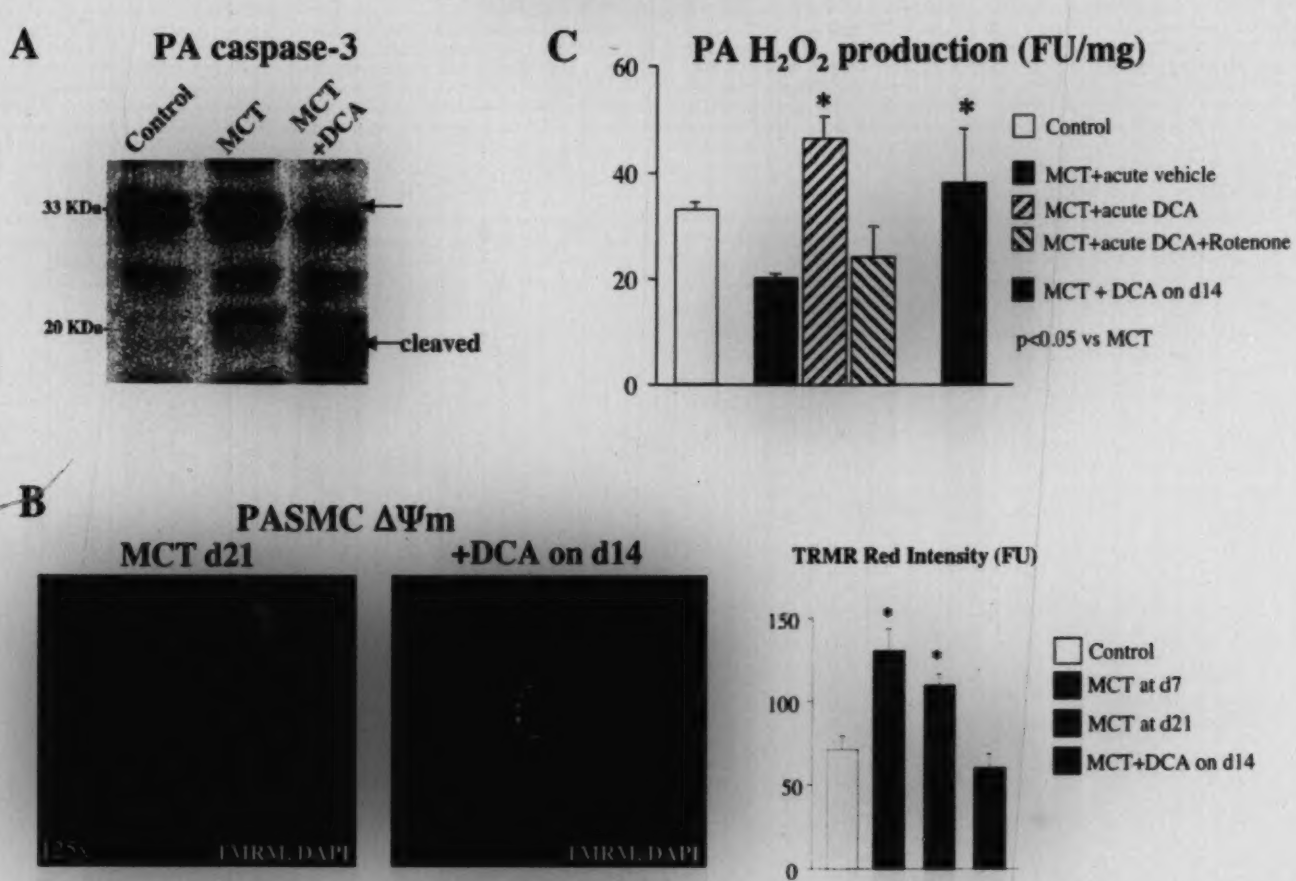

A
PA caspase-3
Control
MCT
MCT +DCA
33 KDa
20 KDa
cleaved
C
PA H_2O_2 production (FU/mg)
60
40
20
0
*
*
☐ Control
■ MCT+acute vehicle
▨ MCT+acute DCA
▧ MCT+acute DCA+Rotenone
■ MCT + DCA on d14
p<0.05 vs MCT
B
PASMC ΔΨm
MCT d21
+DCA on d14
TMRM, DAPI
TMRM, DAPI
TRMR Red Intensity (FU)
150
100
50
0
*
*
☐ Control
■ MCT at d7
■ MCT at d21
■ MCT+DCA on d14

Figure 2-4

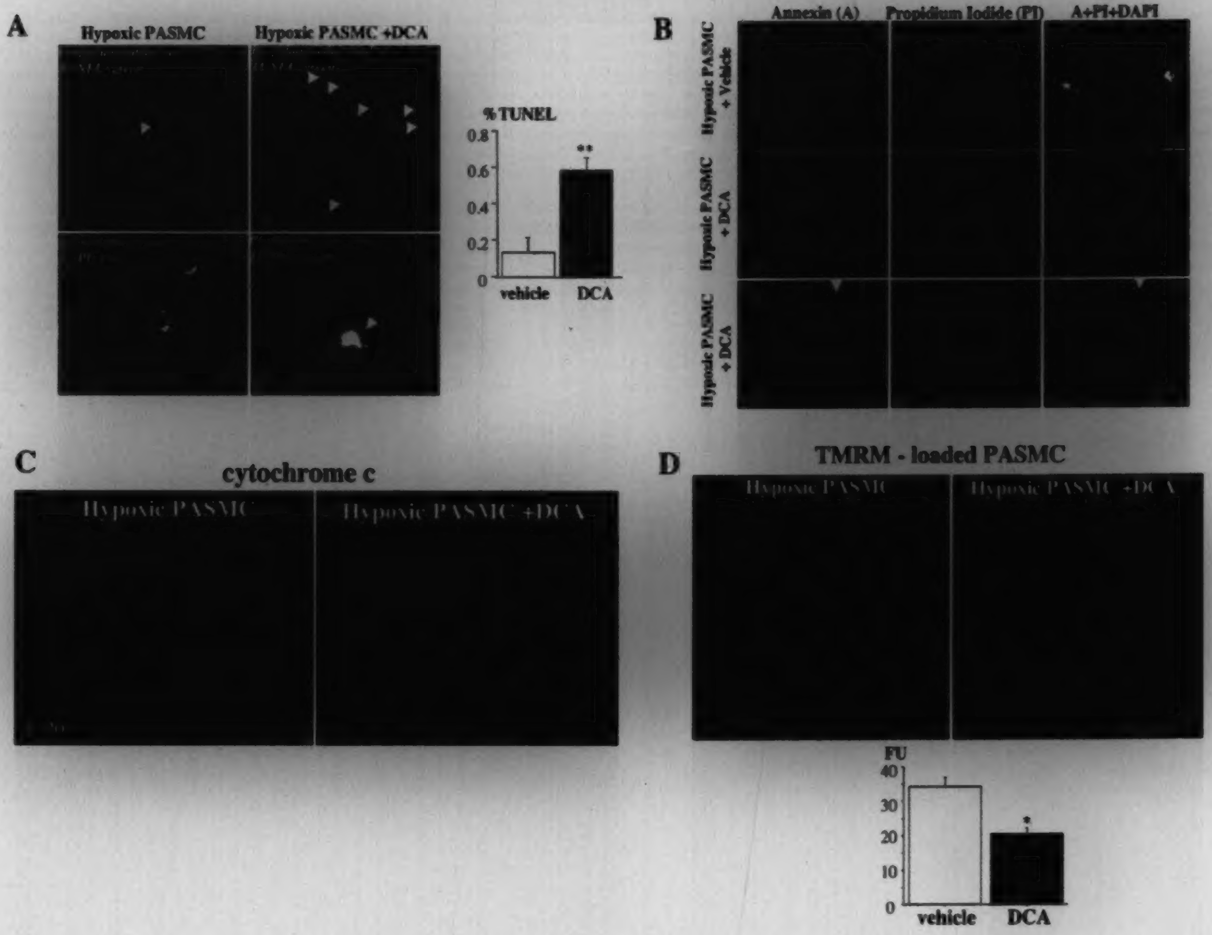

A
Hypoxic PASMC
Hypoxic PASMC +DCA
% TUNEL
0.8
0.6
0.4
0.2
0
**
vehicle
DCA
B
Annexin (A)
Propidium Iodide (PI)
A+PI+DAPI
Hypoxic PASMC + Vehicle
Hypoxic PASMC + DCA
Hypoxic PASMC + DCA
C
cytochrome c
Hypoxic PASMC
Hypoxic PASMC +DCA
D
TMRM - loaded PASMC
Hypoxic PASMC
Hypoxic PASMC +DCA
FU
40
30
20
10
0
*
vehicle
DCA

Figure 2-5

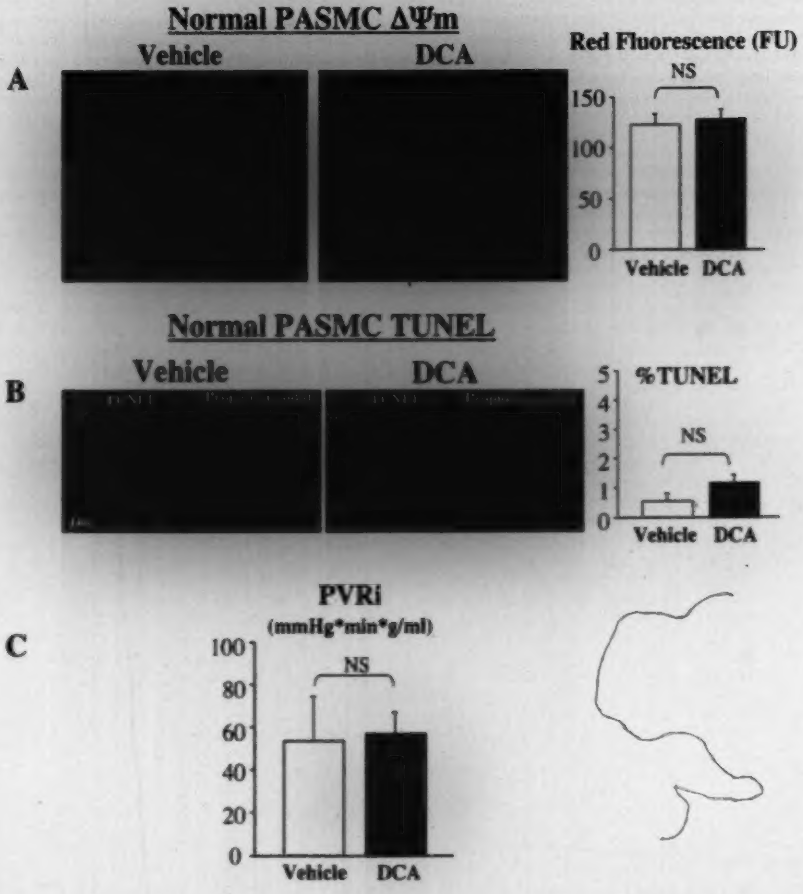

Normal PASMC ΔΨm
A
Vehicle
DCA
Red Fluorescence (FU)
NS
150
100
50
0
Vehicle
DCA
Normal PASMC TUNEL
B
Vehicle
DCA
%TUNEL
5
4
3
2
1
0
NS
Vehicle
DCA
C
PVRi
(mmHg*min*g/ml)
100
80
60
40
20
0
NS
Vehicle
DCA

Figure 2-6

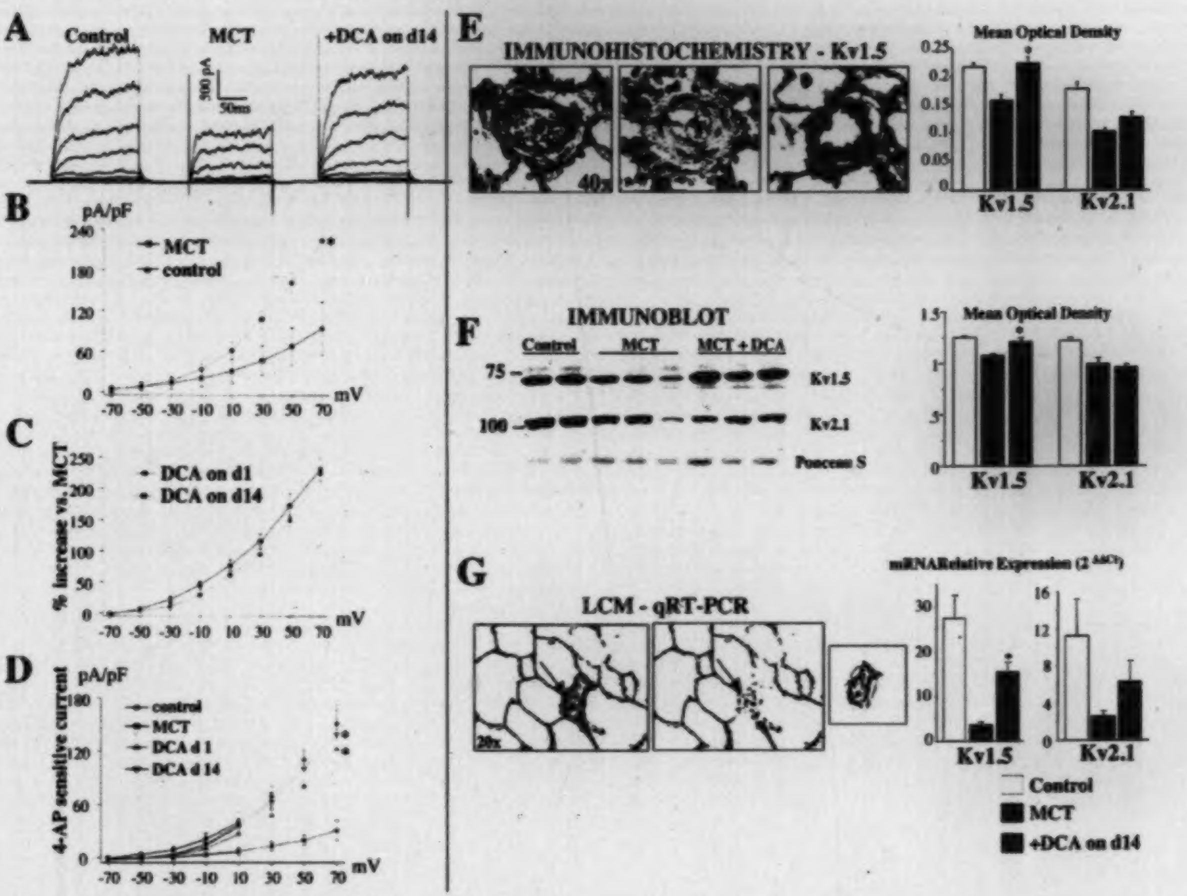

A
Control
MCT
+DCA on d14
500 pA
50ms
B
pA/pF
240
180
120
60
0
MCT
control
-70 -50 -30 -10 10 30 50 70
mV
C
% increase vs. MCT
250
200
150
100
50
0
DCA on d1
DCA on d14
-70 -50 -30 -10 10 30 50 70
mV
D
pA/pF
4-AP sensitive current
180
120
60
0
control
MCT
DCA d 1
DCA d 14
-70 -50 -30 -10 10 30 50 70
mV
E
IMMUNOHISTOCHEMISTRY - Kv1.5
40x
Mean Optical Density
0.25
0.2
0.15
0.1
0.05
0
Kv1.5
Kv2.1
F
IMMUNOBLOT
Control
MCT
MCT + DCA
75
100
Kv1.5
Kv2.1
Ponceau S
Mean Optical Density
1.5
1
.5
0
Kv1.5
Kv2.1
G
LCM - qRT-PCR
20x
mRNARelative Expression (2 ^ΔΔCt)
30
20
10
0
16
12
8
4
0
Kv1.5
Kv2.1
Control
MCT
+DCA on d14

Figure 2-7

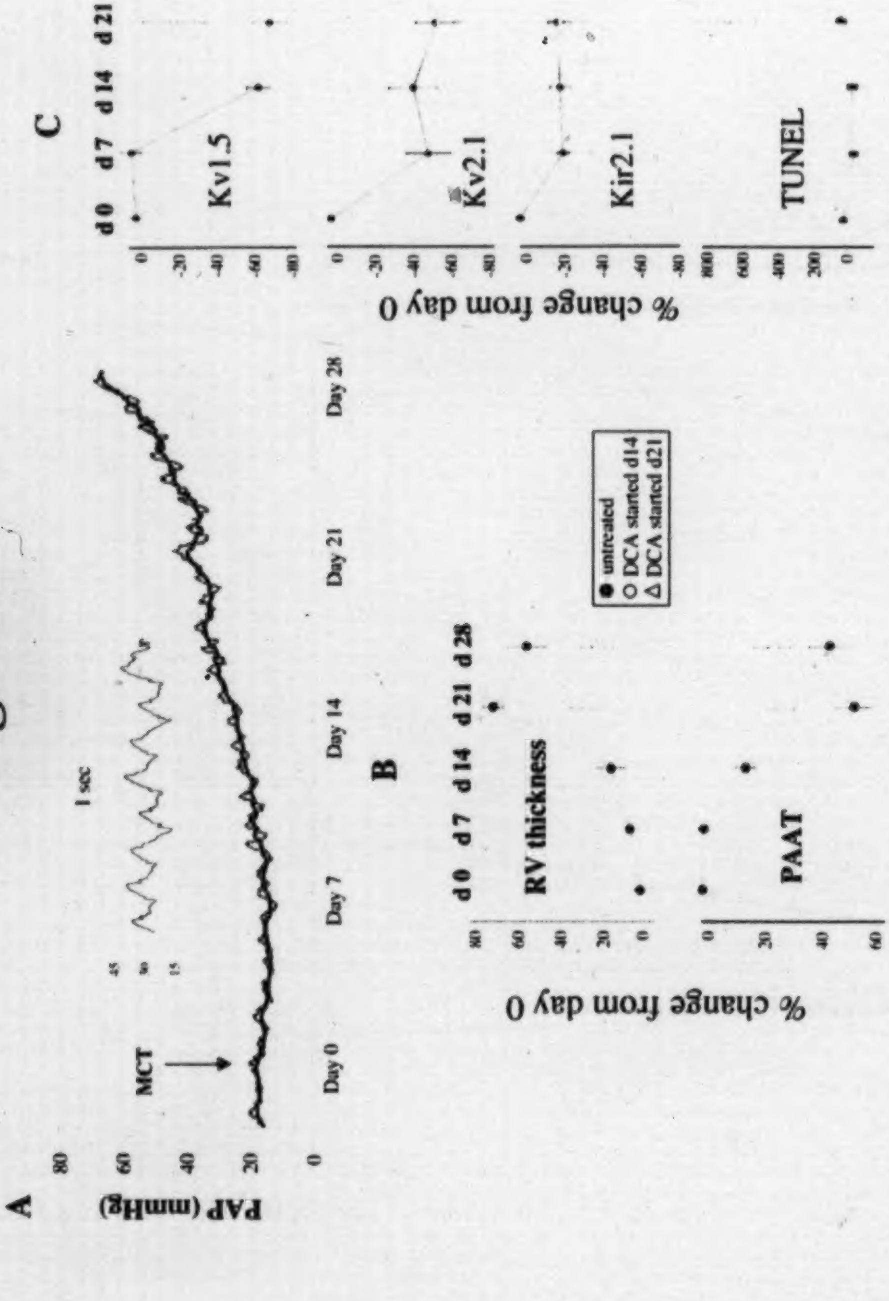
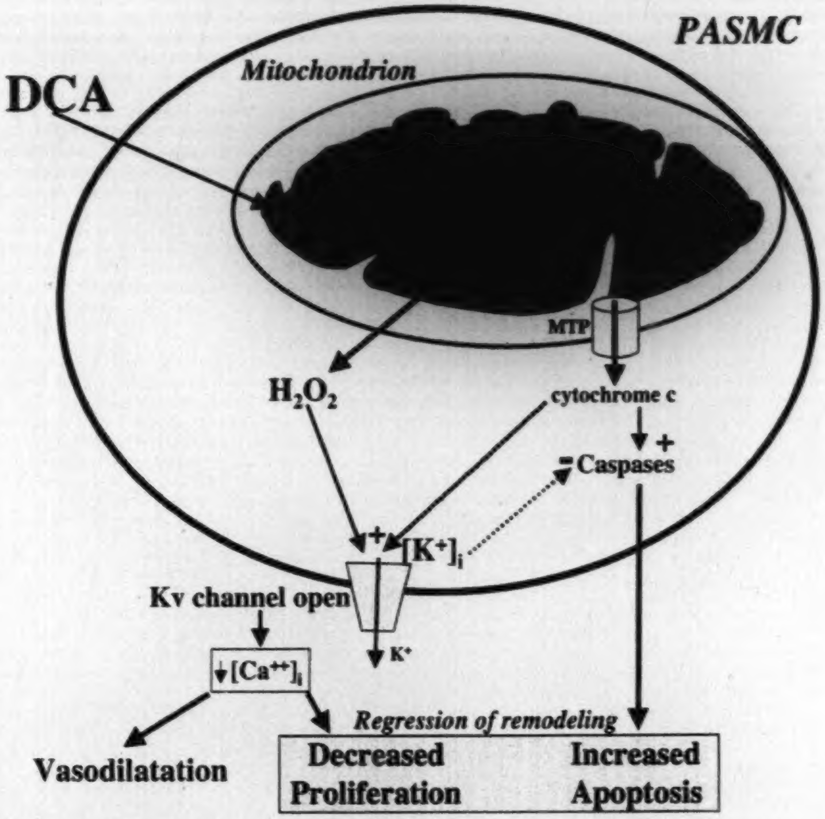

A
PAP (mmHg)
80
60
40
20
0
MCT
1 sec
45
30
15
Day 0
Day 7
Day 14
Day 21
Day 28
B
d 0 d 7 d 14 d 21 d 28
RV thickness
PAAT
% change from day 0
● untreated
○ DCA started d14
△ DCA started d21
C
d 0 d 7 d 14 d 21
Kv1.5
Kv2.1
Kir2.1
TUNEL
% change from day 0
0
-20
-40
-60
-80
800
600
400
200

Figure 2-8


PASMC
Mitochondrion
DCA
MTP
H_2O_2
cytochrome c
+
–
Caspases
+
+
$[K^+]_i$
Kv channel open
K^+
$\downarrow[Ca^{++}]_i$
Vasodilatation
Regression of remodeling
Decreased Proliferation
Increased Apoptosis

Gene therapy targeting survivin selectively induces pulmonary vascular apoptosis and reverses pulmonary arterial hypertension

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Role of M. S. McMurtry

Dr. McMurtry performed the animal experiments and hemodynamic studies, as well as the histology and RV remodeling experiments. He performed the qRT-PCR, LCM, and immunohistochemistry. The immunoblots, confocal microscopy and electrophysiology experiments were performed by technicians with assistance from Dr. McMurtry. All data was collected, integrated and analyzed by Dr. McMurtry.

Citation for this article: J. Clin. Invest. 115:1479–1491 (2005).

Abstract

Pulmonary arterial hypertension (PAH) is characterized by genetic and acquired abnormalities that suppress apoptosis and enhance cell proliferation in the vascular wall, including downregulation of the bone morphogenetic protein axis and voltage-gated K+ (Kv) channels. Survivin is an "inhibitor of apoptosis" protein, previously thought to be expressed primarily in cancer cells. We found that survivin was expressed in the pulmonary arteries (PAs) of 6 patients with PAH and rats with monocrotaline-induced PAH, but not in the PAs of 3 patients and rats without PAH. Gene therapy with inhalation of an adenovirus carrying a phosphorylation deficient survivin mutant with dominant-negative properties reversed established monocrotaline-induced PAH and prolonged survival by 25%. The survivin mutant lowered pulmonary vascular resistance, RV hypertrophy, and PA medial hypertrophy. Both in vitro and in vivo, inhibition of survivin induced PA smooth muscle cell apoptosis, decreased proliferation, depolarized mitochondria, caused efflux of cytochrome c in the cytoplasm and translocation of apoptosis-inducing factor into the nucleus, and increased Kv channel current; the opposite effects were observed with gene transfer of WT survivin, both in vivo and in vitro. Inhibition of the inappropriate expression of survivin that accompanies human and experimental PAH is a novel therapeutic strategy that acts by inducing vascular mitochondria-dependent apoptosis.

Introduction

Pulmonary arterial hypertension (PAH) is a disease of the pulmonary vasculature, defined by an elevated pulmonary vascular resistance (PVR), which eventually leads to right heart failure and premature death. The cause remains unknown, and available treatments are limited, expensive, and often associated with significant side-effects[1]. In PAH, the pulmonary arteries (PAs) manifest pathological proliferative vascular remodeling that includes cellular proliferation in both the intima and the media and muscularization of the normally thin-walled distal PAs[1]. Endothelial dysfunction results in an increase in the ratio of endothelial-derived vasoconstrictors to vasodilators. This imbalance has been the basis of therapies over the past several years; for example, exogenous delivery of vasodilating prostaglandins or blockade of the endothelin axis. However, less that 10% of the patients respond to selective pulmonary vasodilators. While vasoconstriction contributes, especially early in PAH, the obstructive vascular remodeling is the major cause of the elevated PVR and ultimately the right heart failure, which in turn accounts for the 50% 5-year mortality in this disease[1]. There is now a shift in the interest of the scientific community, focusing on therapies aiming to reverse the proliferative remodeling in PAH[2].

Several abnormalities that have been described in PAH contribute to a resistance to apoptosis and a proliferation/apoptosis imbalance within the vascular wall and might explain the PA remodeling: First, in a subset of PAH patients, germ-line and acquired loss-of-function mutations have been described in bone morphogenetic protein receptor 2 (BMPR2)[3, 4]. Activation of the BMPR2 axis leads to suppression of proliferation and activation of apoptosis in normal PA SMCs (PASMCs)[5] but not in PASMCs from patients with PAH[6]. Second, gene microarray studies show that in patients with PAH, there is dysregulation of mediators of apoptosis in the PA wall that favors suppression of apoptosis. For example, bcl-2 is upregulated in PAH [7]. Third, specific PASMC voltage-gated K+ (Kv) channels, such as Kv1.5, are downregulated in both animal models[8, 9] and human PAH[10]. By controlling the membrane potential in PASMCs[11], Kv channels regulate the opening of the voltage-gated L-type Ca++ channels. When Kv channels are inhibited, the influx of Ca++ causes both PASMC contraction and increased proliferation rates[12]. This is further enhanced by the upregulation of transient receptor potential channel genes, which encode for Ca++ channels during proliferation and were recently described in the PASMCs of patients with PAH[13]. Furthermore, by blocking the egress of K+ down its intracellular/extracellular gradient (145:5 mEq), the Kv channel downregulation elevates intracellular K+. Because intracellular K+ tonically inhibits caspases, the inhibition or lack of Kv channels contributes to a resistance to apoptosis[14].

Survivin, a member of the mammalian "inhibitor of apoptosis" family[15], is known to be expressed in essentially all cancers but not in most normal adult cell types [16]. The cell cycle–dependent expression of the survivin gene in mitosis suggests a role for survivin in promoting cell proliferation; however, recent data point to a more selective role of survivin in antagonizing mitochondria-dependent apoptosis (reviewed in ref. 16), and a mitochondrial pool of survivin has recently been shown in cancer cells[17]. The absence of survivin from most healthy tissues makes it very attractive as a target for therapy. Molecular antagonists of survivin, including antisense and dominant-negative mutants, have been consistently associated with induction of apoptosis and inhibition of tumor growth in vivo, without affecting normal cells[16]. Such a mutant is the Thr34→Ala (here described as survivin-M). This mutation prevents a critical phosphorylation of endogenous survivin by the mitotic kinase p34cdc2–cyclin B1. Survivin-M has a 4- to 5-fold accelerated clearance compared with WT survivin and results in a dominant-negative effect by lowering endogenous survivin levels[16]. In addition to inducing apoptosis in cancer cells[16], survivin targeting with survivin-M prevents vascular remodeling in an arterial injury model by inducing apoptosis within the vascular wall[18]. In that study, survivin was absent in quiescent SMCs but was induced in vitro by exposure of aortic SMCs to 20% serum or PDGF or selectively in injured arterial segments in vivo[18]. The pulmonary circulation is very different from the systemic circulation; for example, the pulmonary circulation has low pressure compared with the systemic circulation and constricts to hypoxia, while the systemic circulation dilates[19]. This difference might be in part due to the fact that PASMC mitochondria, important oxygen sensors, are different from the systemic arterial SMC mitochondria: they have lower respiratory rates, are more depolarized, have more manganese superoxide dismutase (MnSOD), and produce more hydrogen peroxide[20]. The mitochondria-produced hydrogen peroxide can activate both Kv channels[21, 22] and guanylate cyclase[23], thereby causing pulmonary vasodilatation. By controlling both vascular tone and apoptosis[24], mitochondria are potentially important in the etiology and therapy of vascular disease, but their role in PAH is not known. Here we investigated a potential role for survivin in PAH. We found that survivin is expressed in remodeled resistance PAs, but not normal PAs, from PAH patients and rats with monocrotaline induced PAH (MCT-PAH), a widely used model of PAH. Inhaled adenoviral gene therapy with survivin-M reverses MCT-PAH. The therapeutic effect of inhibition of survivin is achieved by induction of mitochondria-dependent apoptosis in PASMCs. Interestingly, this is also associated with activation of Kv channels.

Methods

Human studies. Informed consent was obtained according to the principles outlined in the Declaration of Helsinki. Specimens of lung tissue were obtained either from explanted lungs of patients undergoing lung transplantation (PAH, secondary PH), transplant donors (controls) or from wedge resections for lung tumor (controls). Specimens were either flash frozen or paraffin embedded and sectioned for further study.

Experimental protocol. All experiments were conducted with the approval of the University of Alberta Animal Policy and Welfare Committee. Age- and weight-matched male Sprague-Dawley rats (250–300 g) were used. In the reversal protocol, rats were randomized to: control (sham saline injection, n = 7), 60 mg/kg MCT s.c. (n = 20; Sigma-Aldrich), MCT+Ad-GFP (n = 10), and MCT+Ad-GFP-S-M (n = 20). PFUs (5 × 108) of either Ad-GFP or Ad-GFP-S-M was given via intratracheal nebulization on day 13 after MCT, as previously described[9]. All rats underwent hemodynamic and echocardiography studies, and all are included in the mean data presented. In the prevention protocol, rats received the gene therapy at the same time as MCT, i.e., day 1. Rats (n = 6 per group) were randomized in MCT, MCT+Ad-GFP, and MCT+Ad-GFP-S-M groups and were studied on days 21 and 22. In the induction protocol, normal rats were treated with inhaled saline (control, n = 6) or Ad-GFP-S (n = 6) and were studied on days 14 and 15, in order to determine whether WT survivin would induce PAH.

Echocardiography and hemodynamics. RV free wall thickness and PA Doppler signals were measured in the parasternal short axis view using a Sonos 5500 Echo machine with 15-MHz probes (Philips). Rats were then anesthetized with ketamine (60 mg/kg i.p.) and xylazine (20 mg/kg i.p.) and placed on a heated table. Invasive left heart catheterization (carotid pressure and LV end-diastolic pressure [LVEDP]) and right heart catheterization were performed using high-fidelity Millar catheters (Millar Instruments Inc.); cardiac output was measured using a thermodilution probe (ADInstruments) [8, 9, 26]. PVRi was calculated as (mean PA – LVEDP)/cardiac index, and systemic vascular resistance index as (mean arterial pressure – right atrial pressure)/cardiac index.

Telemetry. A Data Sciences International system was used for telemetry[27]. The implanted sensor is a fluid-filled catheter (0.7 mm in diameter, 10 cm long) connected to a pressure transducer. Systolic pressure, diastolic pressure, and mean PA pressure were recorded for 1 minute every 4 hours[26].

Morphometric analysis of RVs and PAs. RVH was measured as RV/(LV + septum) weight ratio at sacrifice. Lungs were inflated with formalin, fixed overnight, and embedded in paraffin. Tissue was stained with H&E or antivWF antibody. Five rats per group were studied, and from each rat at least 2 separate lung sections were examined. Resistance PAs (20–200 μm) chosen randomly from low-power fields were analyzed (approximately 60 arteries per group; 2–3 slides per rat) by 2 blinded investigators using Image-Pro Plus software (Media Cybernetics) [8, 9, 26]. External diameter (ED) and medial thickness (MT) were measured, and percent medial thickness was calculated as 2

× MT × 100/ED. The total number of intraparenchymal PAs was also measured per low-power field (magnification, ×10).

Cell preparation and culture. Isolated PAs (fourth to fifth division) were mechanically denuded of endothelium and digested by papain (1 mg/ml), DTT (0.5 mg/ml), collagenase (0.6 mg/ml), and BSA (0.6 mg/ml; all from Sigma-Aldrich) for 20 minutes at 37°C. Cells were placed in culture medium supplemented with 10% or 0.1% FBS (Sigma-Aldrich) and 1% antibiotic/antimycotic (Invitrogen Corp.) and grown in culture for 3 days at 37°C. PASMCs in culture were exposed to Ad-GFP-S-M or Ad-GFP viruses. One hundred microliters of 5 × 109 PFUs was used in 60-mm plates. The exposure to virus was 6 hours, and cells were washed, kept in their respective FBS conditions, and studied 48 hours later.

Electrophysiology. Whole-cell recordings were performed using the patchclamping technique as previously described[8, 9, 26]. Cells were voltageclamped at –70 mV, and currents were evoked by 20-mV steps from –70 mV to +70 mV using 200-ms pulses. Data were recorded and analyzed using pCLAMP 9 and Clampfit 9 (Axon Instruments). Whole-cell currents divided by cell capacitance gave a measure of current density.

Confocal microscopy. Imaging was performed using a Zeiss LSM 510 confocal microscope, as previously described[8, 9, 26]. Immunostaining was performed on paraffin-embedded tissue using microwave antigen retrieval and the following primary antibodies: anti-survivin (NB 500-201, 1:100; Novus Biologicals Inc.), anti-vWF and anti–smooth muscle actin (for both, 1:40; DakoCytomation), and anti-AIF (10 μg/m; Oncogene Research Products). Secondary antibodies were TRITC (1:200, red; Sigma-Aldrich) and FITC (1:40, green; DakoCytomation). ApopTag apoptosis detection kit (TUNEL stain, Serologicals Corp.) and the PCNA antibody (Dako-Cytomation) were used according to the manufacturer's instructions. BrdU was injected (100 mg/kg i.p.) 4 hours before sacrifice, and staining was performed according to the manufacturer's instructions (F. Hoffman-La Roche Ltd.). Counterstaining of nuclei with DAPI (300 nM; Invitrogen Corp.) was performed for 10 minutes at 20°C. All were mounted with ProLong antifade compound (Invitrogen Corp.). Quantification of images (percentage of nuclei positive for TUNEL or PCNA) was done using Image-Pro Plus software by blinded investigators[26]. Measurement of the mitochondrial membrane potential in live PASMCs was performed using TMRM, as previously described[20, 26].

Immunoblotting. Immunoblotting was performed in pooled PAs (from 3 rats per group, 25 μg protein) for survivin (1:1,000; Novus Biologicals Inc.) and in treated PASMCs (50 μg protein) for caspase assays (caspase-3, 1:500; Upstate; and caspase-9, 1:500; EMD Biosciences Inc.) as previously described[26].

Laser-capture microdissection. Lungs were inflated with and embedded in OCT, flash-frozen, and cut in 10-μm sections using a Leica CM 1850 cryostat (Leica Microsystems Inc.). HistoGene slides and dehydration/staining reagents were used according to the manufacturer's instructions (Arcturus). Laser-capture microdissection was performed using the PixCell II system (Arcturus) as previously described[9, 26].

Quantitative RT-PCR. Samples were added to a microwell plate, along with TaqMan probes and reagents, and quantitative RT-PCR was performed using the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Probes used included survivin, Kv1.5, bcl-2, and 18S as a housekeeping gene (all from Applied Biosystems). Results for relative expression are presented as 2ΔΔCt as previously described[9, 20, 26, 44].

Statistics. Values are expressed as the mean ± SEM. Kruskal-Wallis test or ANOVA was used as appropriate. Fisher's probable least-significant difference test was used for post hoc analysis (StatView 4.02; SAS Institute Inc.). For survival analysis, a cohort of 20 MCT-treated rats and 20 Ad-GFP-S-M–treated rats were prospectively followed for 4 weeks and sacrificed after hemodynamic studies on day 28–30 after MCT. Spontaneous deaths were counted as events, and rats undergoing planed hemodynamic studies and sacrifice were censored. Time-to-event data were plotted using the Kaplan-Meier method, with differences evaluated using the log-rank test[26]. A P value less than 0.05 was considered statistically significant.

Results

Survivin is expressed in the PAs from patients with PAH but not in the PAs from patients without PAH. We performed immunohistochemistry in lungs from 10 patients: 6 with PAH, 1 with pulmonary hypertension due to thromboembolic disease, and 3 without pulmonary hypertension (Figure 1 and Table 1). Survivin was expressed in the media and neointima of remodeled resistance PAs in all PAH patients studied (Figure 1A). In contrast, survivin was absent in the PAs from 3 patients without pulmonary hypertension and in the remodeled PAs from a patient with secondary pulmonary hypertension due to thromboembolic disease (Figure 1B). Survivin was expressed in most of the small resistance PAs and often in medium-sized PAs. In the majority of the PAs studied, there was colocalization of survivin with smooth muscle actin, as shown by double staining of the slides and multiphoton laser confocal microscopy. In several small PAs, there was also colocalization of survivin with vWF, which suggests that survivin was also expressed in cells with endothelial features in the obliterated lumen of the small remodeled PAs. At times, cells expressing survivin were found among cells not expressing smooth muscle actin, external to the media; these cells were likely fibroblasts (patients 4 and 5; Figure 1 and Table 1).

Survivin was expressed in the PAs from PAH but not normal rats, and its expression temporally paralleled the rise in PA pressure. Survivin was heavily expressed in the media of rats with MCT-PAH, a widely accepted model of PAH. Similarly to the human tissue, survivin was not expressed in the PAs of normal rats (Figure 2A). Heavy survivin expression was seen in rats with severe PAH (21 days after MCT injection). In contrast, light expression of survivin was seen in partially remodeled PAs, and no expression was seen in non-remodeled PAs from rats with mild early PAH (11 days after MCT) (Figure 2A); this suggests that the expression of survivin is positively associated with the progression and severity of PA remodeling.

To further study the time course of survivin expression relative to the progression of PAH, rats were sacrificed at different time points after the s.c. injection of MCT. The rats underwent echocardiography studies immediately prior to sacrifice, in which we measured PA acceleration time (PAAT), a Doppler parameter that has been shown to correlate well with PA pressure in both humans and rats (as the PA pressure rises, PAAT shortens)[25, 26]. In parallel experiments, we monitored the PA pressure continuously after MCT injection in freely moving rats with telemetry catheters placed in the PA [26, 27]. The increase in the PA mRNA for survivin paralleled the increase in PA pressure. Survivin expression peaked at 10 days after MCT injection, and PA pressure started to increase shortly after that time, i.e., approximately 12 days (Figure 2B). Immunoblots of pooled PAs from 3 rats also show that survivin protein expression was increased 10 days after MCT. The timing of the peak of survivin expression at day 10 after MCT is in agreement with the timing of survivin expression in the carotid injury model[18]. In contrast, in this early stage of PAH, expression of bcl-2, an antiapoptotic mediator that is upregulated in patients with late/established PAH[7], was not altered. Expression of Kv1.5, a K+ channel downregulated in both animal[8, 9, 26] and human PAH[28], decreased in parallel to the increase in survivin and preceded the rise in PA pressure (Figure 2B).

Survivin regulates mitochondria-dependent apoptosis and K+ current in PASMCs. To study the effect of survivin on PASMCs in vitro, we established a model where primary cultures of PASMCs, isolated from rat resistance PAs (Figure 3A), were exposed to 10% FBS (a condition that is known to induce survivin expression in arterial SMCs [ref. 18] and promote proliferation) or 0.1% FBS (a "starvation" condition that does not induce survivin expression [ref. 18] and promotes apoptosis). We infected the PASMCs with replication-deficient type 5 adenoviruses encoding both GFP and WT survivin (Ad-GFP-S) or both GFP and survivin-M (Ad-GFPS-M); the expression of both the GFP and the survivin genes was driven by a CMV promoter[18]. At a dose of 5 × 109 PFUs (100 μl in 60-mm plates) we achieved infection rates greater than 80% (Figure 3B). Transfer of WT survivin with Ad-GFP-S in "starved" PASMCs (0.1% FBS) suppressed apoptosis (measured by TUNEL and DAPI staining) and promoted cell proliferation (measured by expression of the proliferating cell nuclear antigen, PCNA). In contrast, gene transfer of the survivin mutant with Ad-GFP-S-M in PASMCs exposed to 10% FBS induced apoptosis and decreased PCNA expression (Figure 3, C and D).

We then measured mitochondrial membrane potential using tetramethylrhodamine methyl-ester (TMRM) in PASMCs[26] infected with Ad-GFP-S-M versus Ad-GFP-S. Infection with Ad-GFP-S-M caused significant mitochondrial depolarization (decreased TMRM red fluorescence), compared with noninfected PASMCs from the same plate (Figure 4A). In contrast, gene transfer of WT survivin with Ad-GFP-S caused mitochondrial hyperpolarization, an apoptosis-resistance state. That mitochondria-dependent apoptosis was induced in these PASMCs was also shown by the activation of caspases using immunoblots and cleavage assays; infection with Ad-GFP-S-M, but not with Ad-GFP-S, activated both caspase-9 and caspase-3 (Figure 4A).

Further exploring the mechanism of mitochondria-dependent apoptosis in our model, we performed fluorescence immunocytochemistry to determine whether cytochrome c leaks into the cytoplasm after the mitochondria depolarization. In all PASMCs infected with Ad-GFP-S-M, the cytochrome c staining showed a diffuse, homogeneous pattern, suggesting leakage to the cytoplasm. In contrast, infection with Ad-GFP-S did not cause cytochrome c release, as shown by the punctate, mitochondrial staining pattern (Figure 4B). Infection with Ad-GFP did not alter the mitochondrial membrane potential and did not cause efflux of cytochrome c. We also studied another important mediator of mitochondria-dependent apoptosis, apoptosis-inducing factor (AIF), which causes caspase-independent apoptosis via its nuclease activity in the nucleus[29]. In the PASMCs infected with Ad-GFPS-M, but not in those infected with Ad-GFP-S, AIF was translocated into the nucleus (Figure 4C).

Cytochrome c not only activates caspases but has recently been shown to activate Kv channels in PASMCs, before inducing apoptosis[30]. Since intracellular K+ tonically inhibits caspases, the decrease in intracellular K+ that follows Kv channel activation further promotes apoptosis [14]. Concordant with the mitochondrial depolarization and cytochrome c release, the Ad-GFPS-M–infected PASMCs had a significant increase in PASMC K+ current; conversely, Ad-GFP-S–infected PASMCs had a decrease in K+ current, compared with noninfected cells, as determined by GFP fluorescence (Figure 5A). The efflux of K+ from the cells that follows the K+ channel opening contributes to the osmotic shrinkage of cells, an early marker of apoptosis. As shown by the measurement of PASMC capacitance, the "proapoptotic" Ad-GFPS-M–infected PASMCs were smaller than the non-infected controls (Figure 5A). Ad-GFP-S-M did not alter the K+ current in "serum-starved" PASMCs; this was expected, since the dominant-negative construct would have no effect in a state in which endogenous survivin is absent. On the other hand, Ad-GFP-S further decreased K+ current in PASMCs exposed to 10% FBS.

Survivin inhibition in vivo improves pulmonary hemodynamics and vascular remodeling and prolongs survival in established MCT-PAH. We delivered the survivin mutant selectively to resistance PAs in the lungs of MCT-PAH rats 12–13 days after injection, when endogenous expression of survivin peaks (Figure 2B), and studied them 2 weeks later. We nebulized the Ad-GFP-S-M versus a virus carrying GFP only (Ad-GFP) and delivered them intratracheally. We have previously shown that this route of delivery results in transgene expression that is restricted to the lung and that persists for at least 2 weeks[9]. Diffuse GFP immunofluorescence throughout the vascular wall in medium-sized and small PAs confirmed the effective gene transfer (Figure 5B). Since the small resistance PAs control most of PVR, we quantified the expression of GFP in the microvasculature using laser-capture microdissection and quantitative RT-PCR (Figure 5B). We found that gene delivery was effective particularly in the very small (less than 50 μM) PAs. This is expected, since these PAs are surrounded by alveoli and are directly accessible to the viruses.

In order to study the selectivity and safety of our gene delivery method, we measured the expression of GFP and survivin in treated and untreated rats. We showed that the mRNA levels of survivin in normal and MCT-PAH rats were extremely low in the liver, heart, kidney, and muscle but were unexpectedly high in the spleen. The levels of endogenous survivin were also extremely low in the healthy lung but significantly increased in the treated rats. The significance of the high levels of survivin in the spleen, an organ critical for the immune response, is unclear at this point. If our Ad-GFP-S-M virus were to reach the spleen, the inhibition of the endogenous survivin might have deleterious effects. However, our inhaled gene therapy adds another level of selectivity to our approach. Indeed, in the treated rats, GFP was not detected at all in all organs measured, including the spleen, except the lung (Figure 5C). Further supporting the safety and the selectivity of our approach is the blood work listed in Table 2, showing lack of any hematologic, liver, or renal toxicity during the study period. Gene therapy with Ad-GFP-S-M, but not with Ad-GFP, caused an approximately 50% reduction in PVR index (PVRi), essentially normalizing the PVRi in the treated rats (P value in treated versus untreated rats is not significant). This was accomplished without the alteration of systemic vascular resistance, as expected with selective aerosol gene delivery to the lungs (Figure 6A and Table 2).

In order to obtain a more physiologic assessment of the pulmonary

circulation, we also used Doppler echocardiography in intact rats (before the invasive studies) and showed that the Ad-GFP-S-M–treated rats had improved PAAT, in comparison with the untreated and the Ad-GFP–treated rats (Figure 6B). Our PAAT values correlate strongly with our invasively measured PVRi (the higher the PVRi the lower the PAAT; r2 = 0.86, P < 0.0001, n = 57 rats, i.e., all the rats from our reversal protocol).

In order to determine whether our gene therapy reduced the vascular remodeling in MCT-PAH, we studied PA medial hypertrophy. The Ad-GFP-S-M–treated, but not the Ad-GFP–treated, rats showed a significant reduction in the percentage medial thickness in small and medium-sized PAs (Figure 6C). In contrast, our gene therapy did not alter the vessel density; the number of arteries per low-power field (magnification, ×10) was not statistically different among the control, the MCT-PAH, and the Ad-GFP-S-M–treated arteries.

Furthermore, RV hypertrophy (RVH) improved with the Ad-GFP-S-M, but not with the Ad-GFP, gene therapy. The regression of RVH was shown both macroscopically, by measurement of the RV/(LV + septum) ratio, and echocardiographically, by measurement of the RV free wall thickness in vivo (Table 2). Two-dimensional echocardiography also showed that the shift in the interventricular septum seen in the MCT-PAH rats was normalized in the Ad-GFP-S-M–treated rats, confirming effective decrease in PA pressures (Figure 6B). The significant improvement in the pulmonary hemodynamics and RVH may explain the 25% reduction in mortality (Figure 6D), similar to the result of another experimental therapy inducing mitochondria-dependent apoptosis in the same model [26].

It has been suggested that in the MCT model of PAH, endothelial loss occurs early and leads to loss of microvessels and PAH; suppressing apoptosis with cell-mediated gene therapy with angiopoietin-1 leads to prevention of MCT-PAH[31]. In addition to not finding any difference in vessel density, here and in our recent work[26] we could find no evidence of increased apoptosis early in MCT-PAH, although this possibility cannot be excluded at this point. Nevertheless, theoretically, our proapoptotic strategy could exacerbate endothelial cell loss early on and worsen PAH. Therefore, we delivered the Ad-GFP-S-M and Ad-GFP on day 1 of the MCT injection, and we studied the rats 3 weeks later (n = 6 rats per group). The severity of PAH was similar to that seen with the reversal protocol, but there were no differences in the pulmonary and systemic hemodynamics between the Ad-GFP and Ad-GFP-S-M groups ($P < 0.8$). The lack of any effect from our gene therapy on day 1 is in agreement with the lack of significant endogenous survivin expression during the first 10 days after MCT (Figure 2B).

The reversal of vascular remodeling by survivin targeting is caused by induction of apoptosis, suppression of proliferation, and activation of Kv channels in PASMCs. The survivin mutant gene transfer increased apoptosis (measured by TUNEL) and decreased PCNA expression in the PA media, in agreement with our in vitro data (Figure 7A). BrdU incorporation in PA media was decreased in Ad-GFP-S-M compared with Ad GFP–treated rats, consistent with inhibition of cell proliferation (Figure 7B).

In agreement with our in vitro data, freshly isolated PASMCs from the Ad-GFP-S-M–treated, but not the Ad-GFP–treated, rats had increased K+ current, compared with those from the MCT-PAH rats (Figure 7C). The current morphology and sensitivity to 4-aminopyridine (a relatively specific Kv channel blocker at 5 mM) showed that the induced current was conducted by Kv channels (Figure 7C).

To further support the hypothesis that survivin is directly involved in the pathogenesis of PAH, we delivered WT survivin in normal rats and studied them 2 weeks later (n = 5 per group). Inhaled delivery of Ad-GFP-S in healthy rats caused a mild but significant increase in the medial hypertrophy and PA pressure as well as RVH (Figure 8).

Discussion

Here we show that survivin is etiologically associated with the development of PAH, and we open a new window for the treatment of this devastating disease. Survivin is expressed in established human and experimental PAH, but not in normal PAs. Its expression parallels the rise of PA pressure, although it is not known whether this is true in human PAH. Inhibition of endogenous survivin significantly improves PVRi, PA medial hypertrophy, and RVH, prolonging survival by 25%; in contrast, delivery of WT survivin increases PA pressure, medial hypertrophy, and RVH. We provide direct evidence that both in vitro and in vivo survivin targeting induces PASMC mitochondria-dependent apoptosis and is associated with activation of Kv channels. Our inhaled gene therapy approach is highly selective, with transgene expression restricted in the pulmonary circulation; the reversal of PAH is achieved without any hematologic, liver, or renal toxicity.

Survivin is selectively expressed in cancer because of oncogenic transformation[16]. In vascular SMCs in vitro, survivin expression is increased by exposure to serum growth factors like PDGF[18]. MCT is an endothelial toxin, and a single injection results in endothelial injury selectively in the pulmonary circulation[32], since its active toxic metabolite is secreted by the liver. Endothelial damage results in exposure of PASMCs to circulating growth factors, including PDGF, which is increased early in MCT-PAH, before the rise in PA pressure[33], in agreement with the survivin expression profile (Figure 2B). Indeed, MCT-PAH can be prevented by prophylactic preservation of the endothelium, where the early endothelial cell loss is prevented by gene transfer of the antiapoptotic angiopoietin-1 at the time of MCT injection[31]. Delivering the proapoptotic Ad-GFP-S-M on day 1 did not prevent PAH but also did not worsen it compared with Ad-GFP. This was expected, since the lack of endogenous survivin the first week after MCT explains the lack of any effect of the dominant negative survivin mutant delivered with our replication-deficient adenovirus. This also proves that the beneficial effects of the Ad-GFP-S-M in the reversal protocol were not nonspecific but resulted from the inhibition of endogenous survivin, which is expressed during a specific window in the development of PAH, i.e., 10 days after MCT exposure.

Because PAH patients present late in their clinical course, reversal approaches are much more clinically relevant than prevention. Although increased endothelial apoptosis might indeed be a very early feature of PAH, the suppressed apoptosis and proliferative remodeling in the media that occur later and persist in established PAH are a more therapeutically relevant abnormality. The modulation of apoptosis and proliferation by our gene therapy is localized primarily in the media and PASMCs, as suggested by our in vitro data and vascular histology, although effects on the endothelium or fibroblasts cannot be excluded. In the severe cases of human PAH, survivin was often expressed in cells expressing vWF, in addition to PASMCs, in the remodeled small PAs (Figure 1A). The exact origin of these cells in the remodeled PA is not clear (native vascular cells "invading" from the adventitia and the media, versus circulating precursor cells as part of a distorted regenerative response). Nevertheless, apoptosis of any proliferating cell obstructing the lumen might be beneficial. However, this remains a speculation, and more experiments are needed to determine the relative role of PASMCs and endothelial cells in our proposed model.

The exact role, location, and timing of apoptosis in PAH remain unknown. In support of our findings that induction of apoptosis in the PA media is beneficial in established PAH, are recent studies showing that serine-elastase inhibitors[34], simvastatin[35], and Rho-kinase inhibitors[36] cause regression of established MCT-PAH by inducing PASMC apoptosis. Furthermore, very recent findings from our group show that activation of mitochondria-dependent apoptosis in the PA media by the metabolic modulator dichloroacetate (an inhibitor of the mitochondria enzyme pyruvate dehydrogenase kinase) also leads to reversal of MCT-PAH and an increase in survival. In a striking similarity with the survivin-targeting gene therapy, treatment with dichloroacetate caused depolarization of mitochondria and activation of Kv channels, both in vitro and in vivo, and normalized PVR and RVH, without affecting systemic hemodynamics. Taken together, these data suggest that a dysregulation of a mitochondria–Kv channel axis in PAH might be targeted therapeutically with drugs or gene therapies, with similar beneficial effects.

The fact that the pulmonary circulation is selectively diseased in human PAH is a major therapeutic challenge. The majority of drugs targeting the vasculature will, if given systemically, affect the healthy normal circulation as well, thereby limiting efficacy. For example, L-type Ca++ channel blockers are useful in a subset of PAH patients[37] but are often not tolerated at useful doses because they cause systemic vasodilatation and hypotension[1]. Discovery of factors selectively expressed in the PAs, in addition to novel methods of delivering treatment selectively to the pulmonary circulation (such as inhaled delivery of drugs or genes), is critical. The airway administration of a survivin dominant-negative construct satisfies both requirements to ensure selective targeting of the diseased circulation. This is particularly true since survivin is selectively expressed in the PA media in PAH, but absent in quiescent systemic vascular SMCs[18] and normal PAs (Figures 1 and 2 and Table 1).

Our data strengthen the recently proposed view that survivin, in addition to the effects on cell cycle and cell proliferation, also regulates apoptosis[16,17]. We show that overexpression of WT survivin hyperpolarizes whereas survivin mutant depolarizes PASMC mitochondria, an initiating event in mitochondria-dependent apoptosis (Figure 4). We also show that this mitochondria depolarization is associated with a leak of cytochrome c and AIF in the cytoplasm. Cytochrome c is a known activator of effector caspases and can also activate PASMC Kv channels[30]. Furthermore, depolarized PASMC mitochondria are known to produce more hydrogen peroxide than healthy mitochondria[20, 26, 38]. At low doses (as produced in vivo), hydrogen peroxide is a pulmonary vasodilator and K+ channel opener[21–23]. The leakage of cytochrome c in the cytoplasm and the increased hydrogen peroxide production might explain the increase in the Kv channel current that we observed in PASMCs both in vitro and vivo (Figure 5A and Figure 7C). This activation of K+ currents that results from survivin targeting is likely to be important in PAH, which is associated with selective inhibition of Kv channels and is improved by exogenous delivery of Kv1.5[9]. The Kv channel activation associated with survivin targeting is unexplored in cancer, where there is also recent evidence that overexpression of K+ channels can induce apoptosis and decrease proliferation in malignant cells[39].

Since survivin expression allows the cells to enter a proliferative phase, inhibition of survivin will result in selective apoptosis of the proliferating PASMC compartment in the vascular wall, sparing the quiescent cells. In other words, it is the subset of PASMCs that express PCNA or take up BrdU that will become apoptotic in the survivin mutant–treated animals. This explains our observation that, both in vivo and in vitro, the survivin mutant–treated vessels had decreased PCNA and BrdU staining (Figures 3 and 7). That survivin targeting selectively causes apoptosis of the proliferating PASMCs is in agreement with the kinetics of apoptosis in cell cycle–synchronized cultures of tumor cells exposed to the same survivin mutant[40].

We offer the following model for the role of survivin in the vascular biology of PAH (Figure 9): Survivin mutations, similar to those observed in oncogenic transformation[41], might occur in some patients with PAH, resulting in spontaneous survivin expression, although this is entirely speculative. Voelkel, Tuder, and colleagues have exposed fascinating similarities between cancer and PAH, by showing that the proliferating cells in the neointimal plexogenic PAH lesions are monoclonal[42]. Alternatively, survivin expression may be induced following endothelial damage, which is widely recognized as a critical early event in the pathogenesis of PAH[31,43]. Endothelial damage would allow direct exposure of PASMCs to circulating growth factors that induce survivin expression. In addition to facilitating the PASMC transition to a proliferative state, survivin would inhibit apoptosis by hyperpolarizing mitochondria. This would result in less cytochrome c and H_2O_2 in the cytoplasm, decreasing the tonic activation of Kv channels. The suppression of Kv channel activity would cause an increase in the intracellular K+, further suppressing apoptosis, and, by depolarizing the cell membrane, would increase the opening of the voltage-gated Ca++ channels; this would increase the influx of Ca++, causing vasoconstriction and amplification of proliferative signal pathways.

The proposed causative role of survivin in PAH and the lack of its expression in quiescent cells in the PA wall and the systemic vasculature make this pathway attractive for future PAH therapies. However, PAH is a multifactorial disease, and activation of the survivin axis might be only 1 of several abnormalities that contribute to the development of PAH in a given patient. Nevertheless, the lung selective inhaled gene therapy approach, the lack of systemic toxicity, and its effectiveness in prolonging survival make survivin targeting an attractive candidate therapy for human PAH. Our proposed survivin–mitochondria–Kv channel axis merits further assessment not only in vascular biology but in cancer biology as well.

Acknowledgments

E.D. Michelakis and S.L. Archer are supported by the Canada Foundation for Innovation, the Alberta Heart and Stroke Foundation, the Alberta Heritage Foundation for Medical Research, the Canadian Institutes for Health Research, and the Alberta Cardiovascular and Stroke Research Centre (ABACUS). S.L. Archer is also supported by NIH grant HL071115. E.D. Michelakis is Canada Research Chair in Pulmonary Hypertension; S.L. Archer is Canada Research Chair in Translational Cardiovascular Medicine and Oxygen Sensing.

M.S. McMurtry is supported by the Clinician Investigator program at the University of Alberta and a training grant from Bristol-Myers Squibb Co. D.C. Altieri is supported by NIH grants HL54131, CA78810, and CA90917.

Figure Legends

Figure 3-1 – Survivin is expressed in the PAs of patients with PAH, but not the PAs of patients with secondary pulmonary hypertension or normal PAs. (A) Top: Immunofluorescence confocal microscopy shows survivin expression colocalizing with smooth muscle actin in the media of small and medium-sized PAs from 5 patients with PAH. In some cases (patients 3–5) survivin is expressed in cells both outside and inside the elastic lamina (evident by autofluorescence in patient 3). Bottom: In 2 small PAs shown from a PAH patient, survivin is also expressed; it colocalizes with vWF only in the bottom one, which appears to be more remodeled and have an almost obliterated lumen. (B) Survivin is not expressed in the PAs of 3 patients without PAH (patients 8 and 9; patient 10 not shown). Despite the significant medial hypertrophy shown in patient 7, due to thromboembolic pulmonary hypertension, there is a compete lack of survivin expression, suggesting that it is not a nonspecific feature of the remodeling process. Lack of nonspecific staining by the secondary antibodies in patient 4 is shown in the bottom; this was the case in all the patients shown here. SMA, smooth muscle actin; S, survivin; L, lumen.

Figure 3-2 - Survivin is expressed in the PAs of rats with MCT-PAH, and its expression parallels the rise in PA pressure. (A) Survivin is expressed in the media of resistance PAs from rats with MCT-PAH but not in PAs from control rats. Note the heavy expression of survivin in severe MCT-PAH (21 days after MCT), compared with mild MCT-PAH (12 days after MCT). In the latter, note that a smooth muscle actin-positive

PASMC is heavily expressing survivin in the media of a small PH that shows early medial hypertrophy, whereas no survivin is expressed in another PA from the same field that does not show evidence of medial hypertrophy. Magnification in S+SMA+DAPI, ×75. (B) Survivin expression, measured by quantitative RT-PCR and immunoblots, increases 10 days after MCT injection, prior to the increase of PA pressure as measured by in vivo telemetry (mean PA pressure [PAP] shown) and by echocardiography (PAAT). Kv1.5 expression is decreasing in parallel with survivin, before the rise in pressure, while bcl-2 expression is unchanged.

Figure 3-3 - Efficient adenoviral delivery of phosphorylation-deficient survivin (Ad-GFP-S-M) to PASMCs in vitro reduces proliferation and increases apoptosis. (A) Primary culture of rat PASMCs stains positive for smooth muscle actin but not vWF, indicating no contamination with endothelial cells. (B) Infection of PASMCs with adenoviruses encoding GFP and either WT survivin (Ad-GFP-S) or survivin mutant (Ad-GFP-S-M) was highly efficient, as evidenced by GFP reporter (green fluorescence in the left panels and differential interference contrast [DIC] in the right panels). Note the reduced cellularity of the plate infected with Ad-GFP-S-M, compared with Ad-GFP-S. (C) Cells infected with Ad-GFP-S-M (grown in 10% FBS) show increased TUNEL-positive nuclei and reduced PCNA-positive nuclei, suggesting that they undergo apoptosis and not proliferation. In contrast, cells infected with Ad-GFP-S (grown in 0.1% FBS) show no apoptosis and increased rates of PCNA expression. (D) Mean data for TUNEL- and PCNA-positive nuclei, 48 hours after infection with Ad-GFP-S-M versus Ad-GFP-S; 5 fields studied in each plate, 20 plates per group. $*P < 0.01$.

Figure 3-4 - Ad-GFP-S-M infection induces PASMC mitochondria-dependent apoptosis. (A) PASMCs effectively infected with Ad-GFP-S (green) show slight hyperpolarization of mitochondrial membrane potential (increased TMRM fluorescence) compared with noninfected cells. In contrast, mitochondria of Ad-GFP-S-M–infected cells (but not neighboring noninfected cells) are less red, indicating depolarized mitochondria. Mean data are shown on the right (arbitrary fluorescence units [FU], means from 15 plates per group; $*P < 0.01$). Immunoblots show that, in contrast to expression of WT survivin, expression of survivin mutant in PASMCs grown in 10% FBS induces activation of caspase-9 and caspase-3. (B) PASMCs infected with Ad-GFP-S demonstrate sequestered cytochrome c within mitochondria, as shown by the punctate pattern of staining. In contrast, mitochondria of Ad-GFP-S-M–infected cells show cytochrome c–positive staining diffusely throughout the cell, indicating leakage of cytochrome c from mitochondria into the cytoplasm. Magnification: left and middle panels, ×75; right panels, ×125. (C) In contrast to infection with Ad-GFP-S, infection with Ad-GFP-S-M induces translocation of the mitochondria-based apoptosis-inducing factor (AIF) in the nucleus, where it initiates caspase-independent apoptosis. Magnification, ×100.

Figure 3-5 - Selective expression of survivin mutant in resistance PAs causes an increase in PASMC outward K+ current. (A) In FBS-rich conditions (10% FBS in the medium, a condition known to increase endogenous survivin), infection with Ad-GFP-S-M causes augmentation of K+ currents and decreased capacitance (Cm, a measure of cell size), consistent with apoptosis; the opposite is seen with Ad-GFP-S infection. In contrast, in

serum-deprived conditions (0.1% FBS), infection with Ad-GFP-S causes a decrease in K+ currents, consistent with apoptosis resistance. Since in these conditions endogenous survivin is absent, infection with Ad-GFP-S-M has no effect on K+ current. Cells carrying the transgenes were selected by the green fluorescence. Mean data for current density over voltage are shown on the right (n = 6 cells per group; *P < 0.01 vs. control). (B) Both GFP immunofluorescence microscopy and quantitative RT-PCR of laser-capture-microdissected resistance PAs demonstrate efficient delivery of the transgenes, particularly to the very small (less than 50 μm) resistance PAs (arrows). (C) In our inhaled gene therapy approach, the expression of the transgenes is restricted to the lungs, as shown by the expression of GFP, measured by quantitative RT-PCR. The expression of endogenous survivin in nontreated rats is minimal in all organs studied, except the spleen. Our WT-survivin primer also detects the survivin mutant, as shown by the increased lung signal in the treated rats. Data from 5 rats per group are shown.

Figure 3-6 - Gene therapy of rat MCT-PAH with Ad-GFP-S-M improves hemodynamics, reduces remodeling of the resistance PAs, and prolongs survival. (A) Representative high-fidelity PA pressure tracings and mean data show that Ad-GFP-S-M, but not Ad-GFP, therapy reduces PA pressure and PVRi, without altering systemic hemodynamics. SVRi, systemic vascular resistance index. (B and C) Ad-GFP-S-M, but not Ad-GFP, reduces RV thickness measured in parasternal short axis (see also Table 2) and preserves the normal round shape of the LV. Similarly, Ad-GFP-S-M reduces PAAT. Resistance PA remodeling, as measured by percent medial thickness, is reduced by treatment with Ad-GFP-S-M. RVOT, RV outflow tract; AV, aortic valve; PV, pulmonary

valve. Magnification in C, ×40. (D) Targeting survivin with inhaled gene therapy in MCT-PAH significantly prolongs survival within the study period. *P < 0.05 vs. MCT; †P < 0.05 vs. Ad-GFP.

Figure 3-7 - Ad-GFP-S-M augments apoptosis and Kv current and reduces proliferation within resistance PAs in vivo. (A) The number of TUNEL-positive nuclei (arrows) is increased by Ad-GFP-S-M treatment, while the number of PCNA-positive nuclei is reduced, compared with those in the Ad-GFP–treated and nontreated MCT-PAH rats. *P < 0.05 vs. MCT; †P < 0.05 vs. Ad-GFP. (B) Reduced BrdU staining (green) in resistance PAs of rats treated with Ad-GFP-S-M, compared with Ad-GFP-S (a representative image from 5 rats per group is shown). (C) Freshly isolated PASMCs from rats treated with Ad-GFP-S-M have increased K+ currents, in agreement with our in vitro data (Figure 5A). The sensitivity to 4-aminopyridine (4-AP; 5 mM) and current morphology suggest that the induced current is voltage-gated (Kv). *P < 0.05 vs. Ad-GFP.

Figure 3-8 - Exogenous WT survivin, delivered by Ad-GFP-S in normal rats, induces PAH and medial hypertrophy, within 2 weeks from infection. Magnification, ×10.

Figure 3-9 - Schematic linking mitochondria, survivin, and Kv channels as potential therapeutic targets for the regression of pulmonary vascular remodeling. Cyt c, cytochrome c.

Tables

Table 1 - Information on the patient tissues used for immunohistochemistry studies

Patient	Type	Age/Sex	Severity	Epoprostanol	Material
1	iPAH	31 M	Severe	+	Transplant
2	iPAH	52 M	Severe	+	Transplant
3	PAH-VSD	32 F	Severe	–	Wedge biopsy
4	iPAH	48 F	Severe	–	Transplant
5	PAH-ASD	46 F	Moderate	–	Wedge biopsy
6	iPAH	33 F	Severe	–	Transplant
7	2-PHT[A]	38 M	Severe	–	Wedge biopsy
8	Normal	26 M			Donor
9	Normal 3	8 F			Donor
10	Normal	42 M			Lobectomy[B]

Patient numbers correspond to those in Figure 1, A and B. [A]Secondary PAH due to thromboembolic disease. [B]Benign tumor of the lung. iPAH, idiopathic PAH; PAH-VSD, PAH due to ventricular septal defect; PAH-ASD, PAH associated with atrial septal defect.

Table 2 - Hemodynamic and toxicity data of the survivin-targeting gene therapy in established MCT-PAH

	Control	**MCT**	**+Ad-GFP**	**+Ad-GFP-S-M**
PVRi (mmHg × min × g/ml)	35 ± 6	121 ± 10	117 ± 9	57 ± 3^{A}
SVRi (mmHg × min × g/ml)	269 ± 16	282 ± 8	269 ± 15	254 ± 9
Mean PAP (mmHg)	17 ± 4	40 ± 3	39 ± 2	25 ± 1^{A}
LVEDP (mmHg)	4.3 ± 0.3	4.7 ± 0.2	4.1 ± 0.4	4.8 ± 0.2
Systolic BP (mmHg)	91 ± 1	82 ± 1	80 ± 4	92 ± 2^{B}
Heart rate (beats/min)	260 ± 18	257 ± 7	265 ± 9	245 ± 7^{A}
CI (ml/min/g)	349 ± 25	294 ± 8	299 ± 14	366 ± 8^{A}
RV/(LV + septum)	0.29 ± 0.01	0.51 ± 0.03	0.51 ± 0.02	0.37 ± 0.14^{A}
PAAT (ms)	31 ± 1	18 ± 2	18 ± 1	26 ± 1^{A}
RV thickness (mm)	0.6 ± 0.02	0.9 ± 0.05	0.9 ± 0.04	0.7 ± 0.03^{B}
Hgb (g/l)	150 ± 2	150 ± 6		148 ± 2
wbc	9 ± 3	12 ± 3		10 ± 2
Platelet	1,010 ± 56	1,029 ± 74		1,096 ± 98
AST (U/l)	80 ± 5	70 ± 9		80 ± 2
Creatinine (μM/l)	46 ± 4	52 ± 9		50 ± 13

^{A}P < 0.0001 vs. Ad-GFP; ^{B}P < 0.001 vs. Ad-GFP. PVRi, pulmonary vascular resistance index; SVRi, systemic vascular resistance index; PAP, PA pressure; LVEDP, LV end-diastolic pressure; CI, cardiac index; Hgb, hemoglobin; AST, aspartate aminotransferase.

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monocrotaline induced fatal pulmonary hypertension in rats. *Circ. Res.* 94:385–393, 2004.

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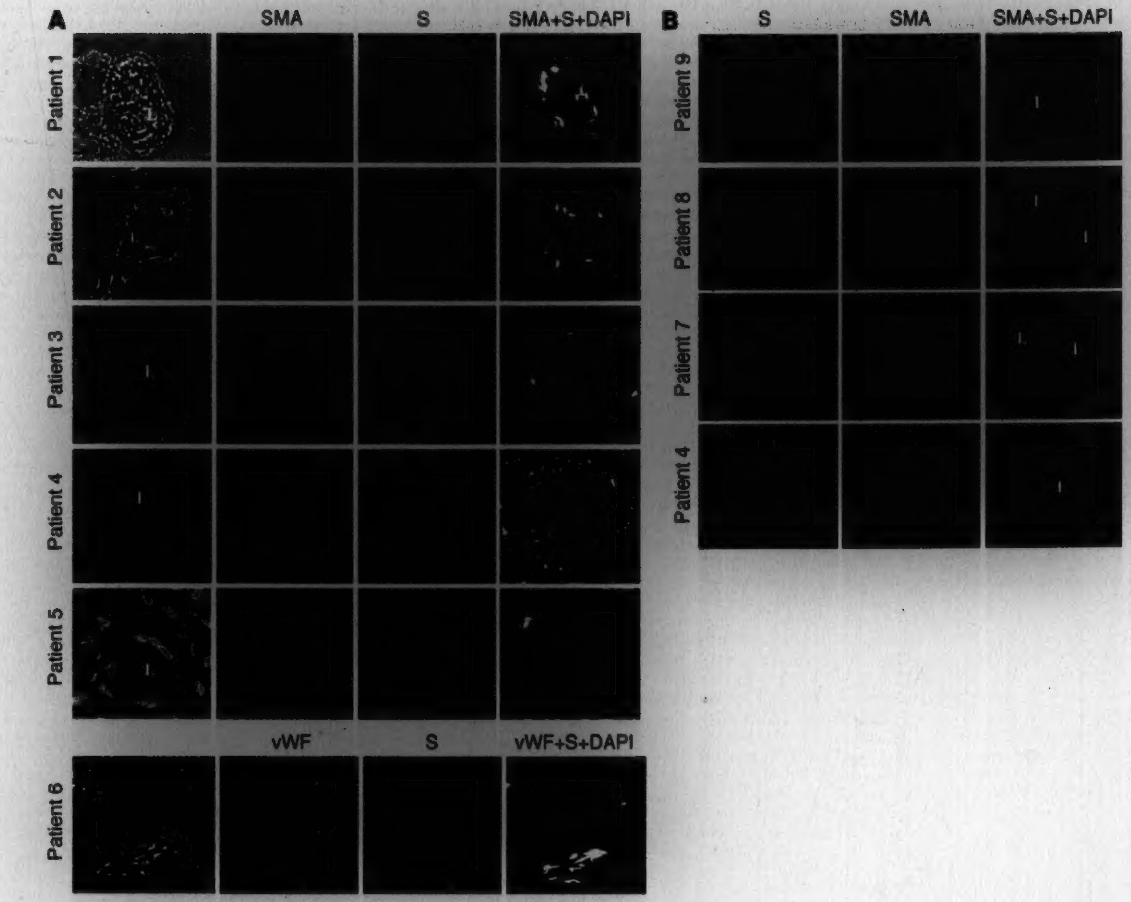
Figure 3-1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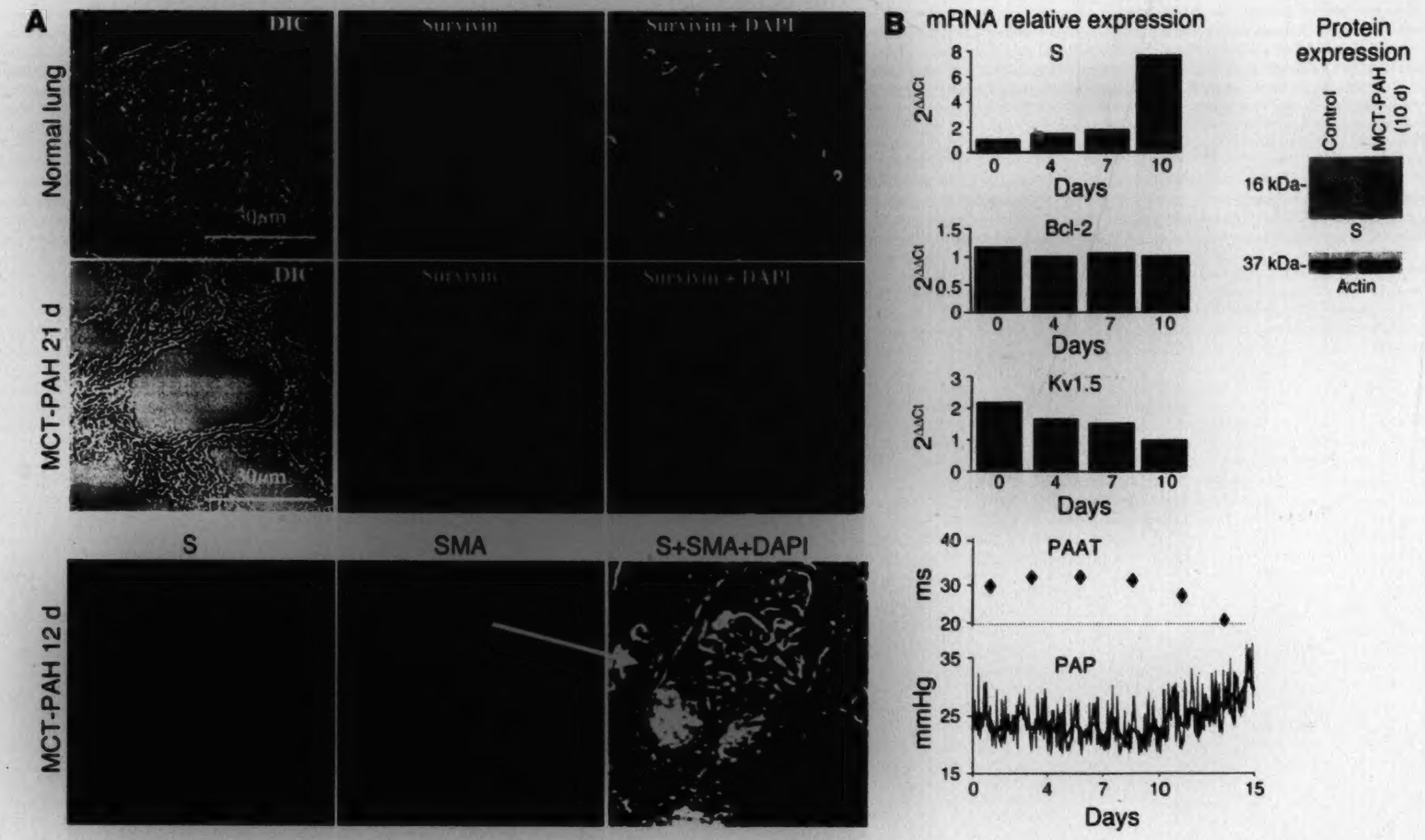
SMA
S
SMA+S+DAPI
Patient 1
Patient 2
Patient 3
Patient 4
Patient 5
vWF
S
vWF+S+DAPI
Patient 6
B
S
SMA
SMA+S+DAPI
Patient 9
Patient 8
Patient 7
Patient 4

Figure 3-2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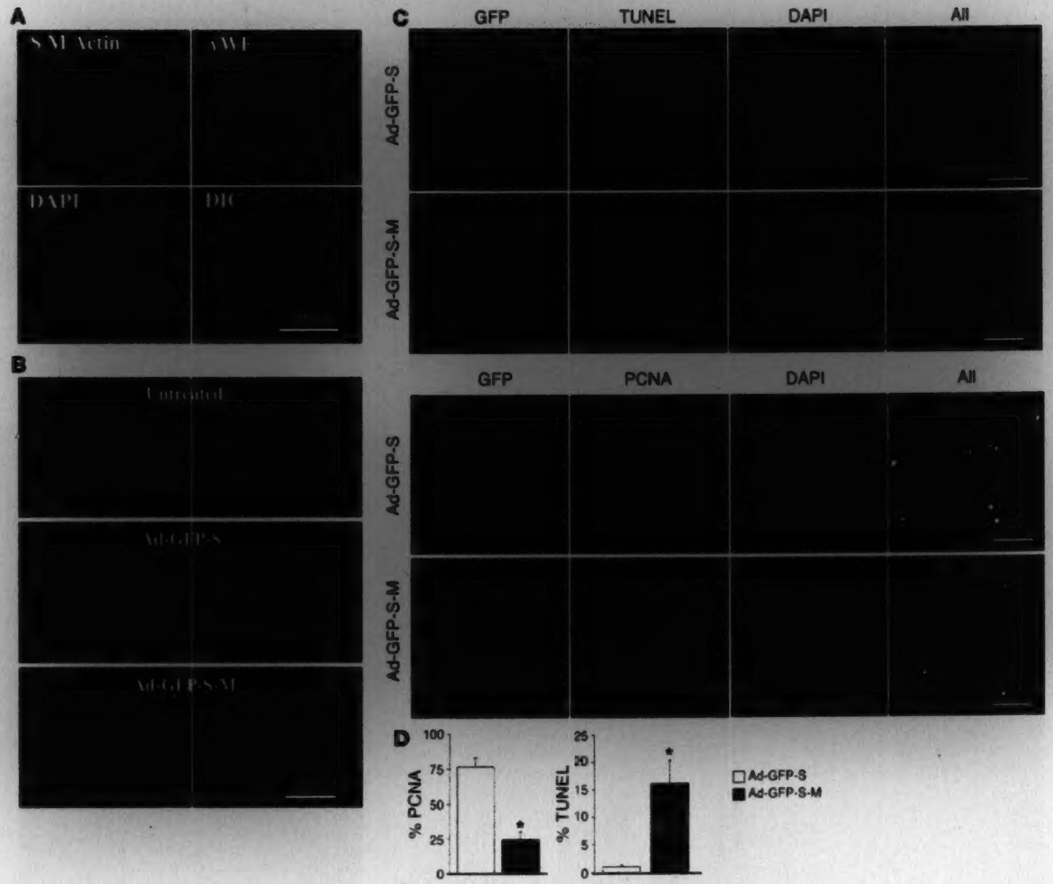
DIC
Survivin
Survivin + DAPI
Normal lung
30μm
MCT-PAH 21 d
MCT-PAH 12 d
S
SMA
S+SMA+DAPI
B
mRNA relative expression
S
Bcl-2
Kv1.5
$2^{\Delta\Delta Ct}$
Days
0
4
7
10
PAAT
ms
PAP
mmHg
15
Protein expression
Control
MCT-PAH (10 d)
16 kDa-
37 kDa-
Actin

Figure 3-3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SM Actin
vWF
DAPI
DIC
B
Untreated
Ad-GFP-S
Ad-GFP-S-M
C
GFP
TUNEL
DAPI
All
Ad-GFP-S
Ad-GFP-S-M
GFP
PCNA
DAPI
All
Ad-GFP-S
Ad-GFP-S-M
D
% PCNA
100
75
50
25
0
*
% TUNEL
25
20
15
10
5
0
*
☐ Ad-GFP-S
■ Ad-GFP-S-M

Figure 3-4

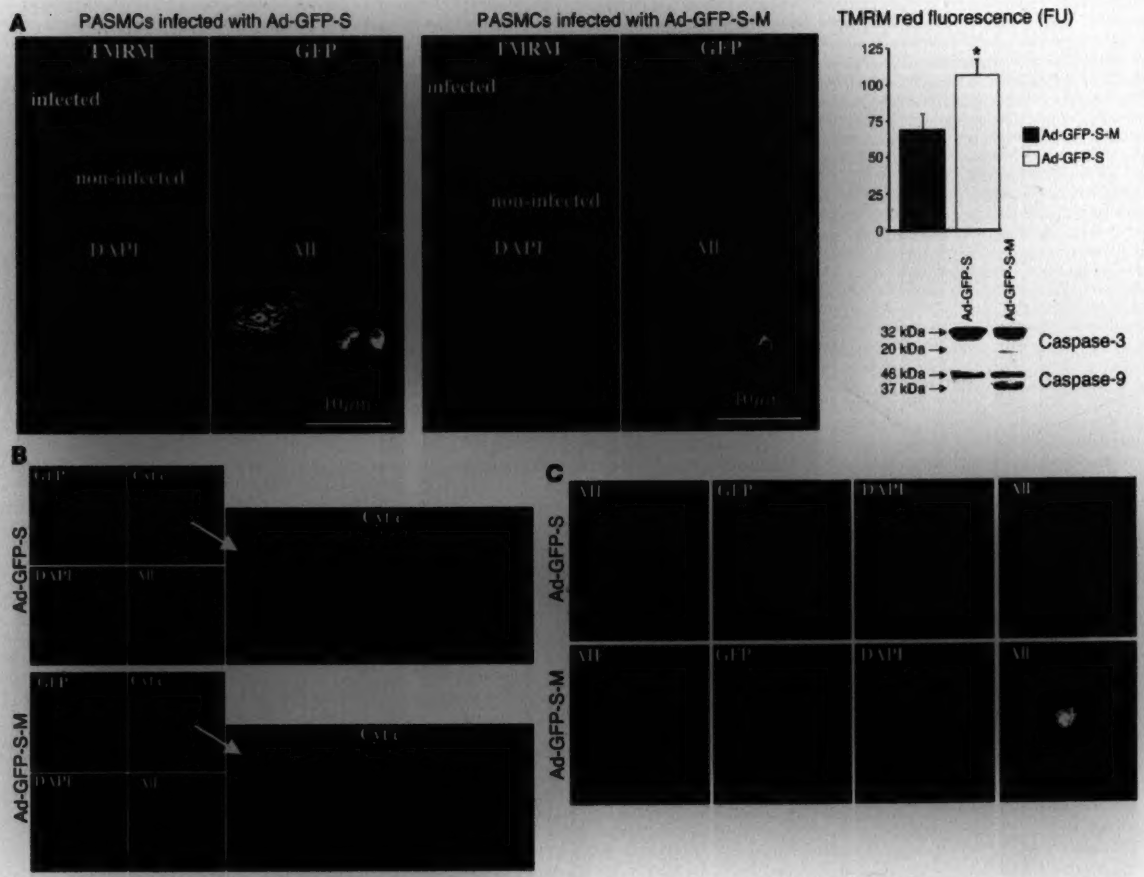

A
PASMCs infected with Ad-GFP-S
PASMCs infected with Ad-GFP-S-M
TMRM
GFP
infected
non-infected
DAPI
All
10μm
TMRM red fluorescence (FU)
125
100
75
50
25
0
*
Ad-GFP-S-M
Ad-GFP-S
32 kDa →
20 kDa →
46 kDa →
37 kDa →
Caspase-3
Caspase-9
B
Ad-GFP-S
Ad-GFP-S-M
GFP
Cyt c
DAPI
All
C
Ad-GFP-S
Ad-GFP-S-M
All
GFP
DAPI
All

Figure 3-5

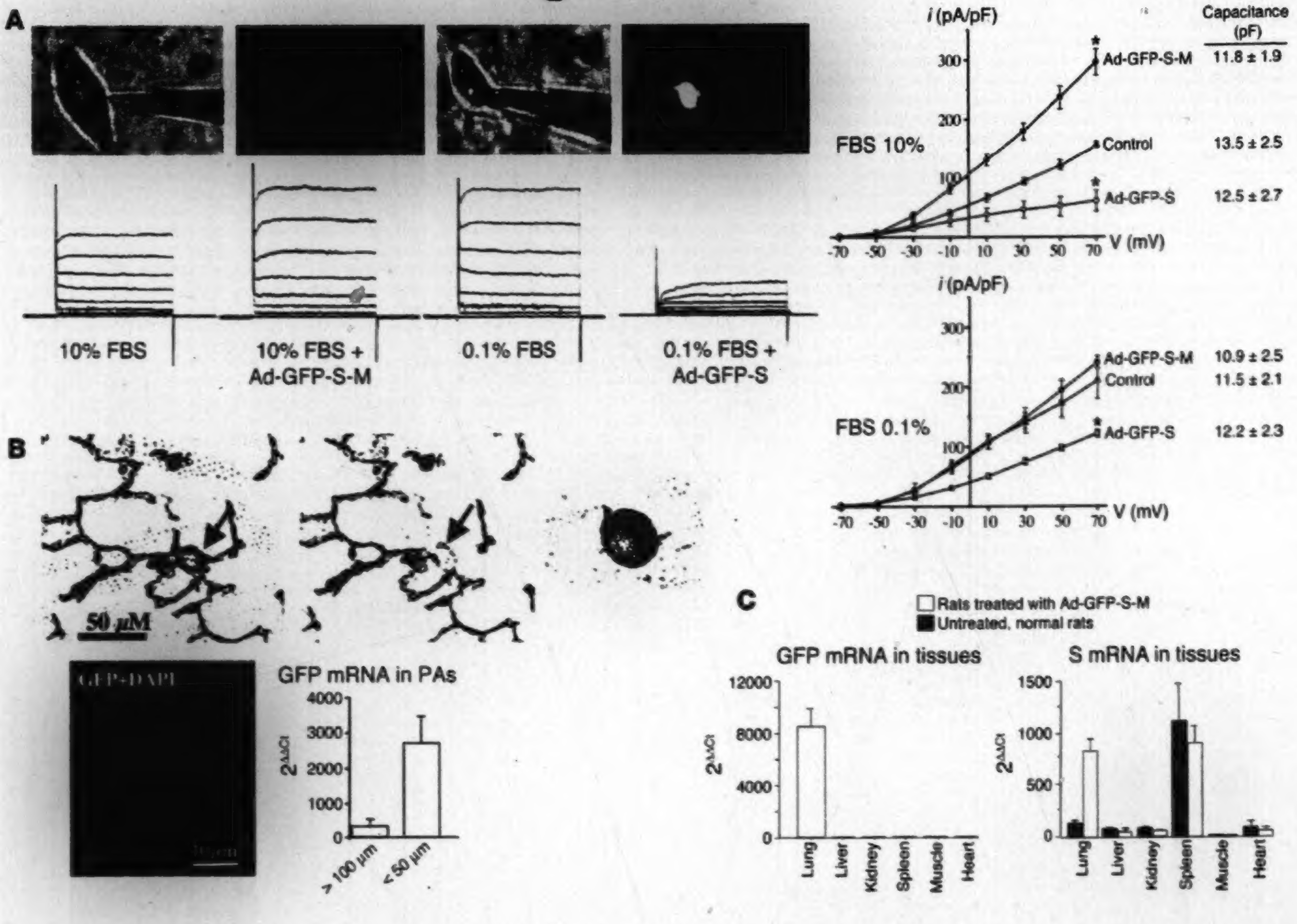
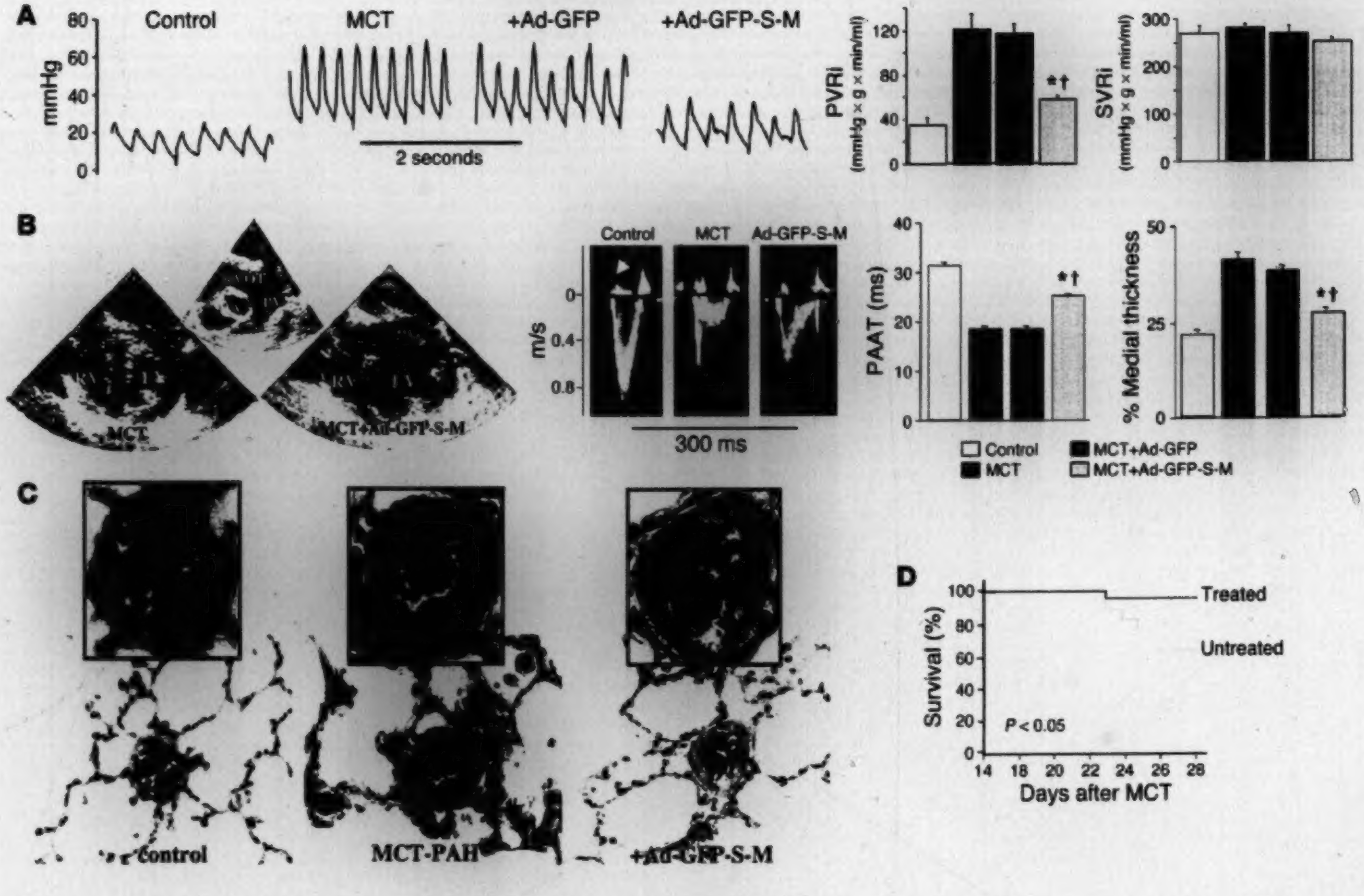

A
10% FBS
10% FBS + Ad-GFP-S-M
0.1% FBS
0.1% FBS + Ad-GFP-S
FBS 10%
i (pA/pF)
300
200
100
-70 -50 -30 -10 10 30 50 70
V (mV)
Ad-GFP-S-M
Control
Ad-GFP-S
*
Capacitance (pF)
11.8 ± 1.9
13.5 ± 2.5
12.5 ± 2.7
FBS 0.1%
10.9 ± 2.5
11.5 ± 2.1
12.2 ± 2.3
B
50 μM
GFP-DAPI
GFP mRNA in PAs
4000
3000
2000
1000
0
$2^{\Delta\Delta Ct}$
> 100 μm
< 50 μm
C
Rats treated with Ad-GFP-S-M
Untreated, normal rats
GFP mRNA in tissues
12000
8000
4000
S mRNA in tissues
1500
1000
500
Lung
Liver
Kidney
Spleen
Muscle
Heart

Figure 3-6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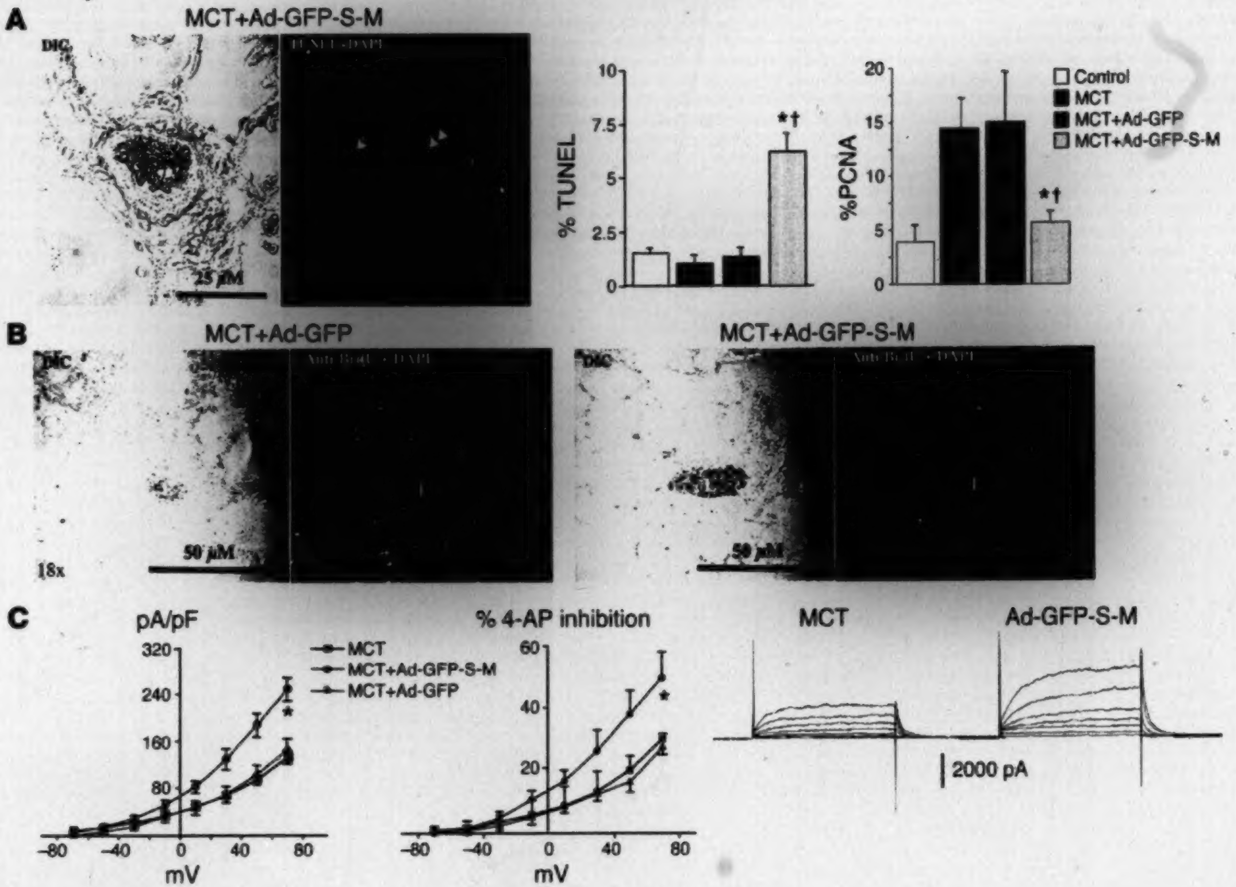
mmHg
80
60
40
20
0
Control
MCT
+Ad-GFP
+Ad-GFP-S-M
2 seconds
PVRi
(mmHg × g × min/ml)
120
80
40
0
*†
SVRi
(mmHg × g × min/ml)
300
200
100
0
B
MCT
MCT+Ad-GFP-S-M
Control
MCT
Ad-GFP-S-M
m/s
0
0.4
0.8
300 ms
PAAT (ms)
40
30
20
10
0
*†
% Medial thickness
50
25
0
*†
Control
MCT
MCT+Ad-GFP
MCT+Ad-GFP-S-M
C
Control
MCT-PAH
+Ad-GFP-S-M
D
Survival (%)
100
80
60
40
20
0
Treated
Untreated
P < 0.05
14
18
20
22
24
26
28
Days after MCT

Figure 3-7



A
MCT+Ad-GFP-S-M
DIC
TUNEL+DAPI
25 μM
% TUNEL
10
7.5
5
2.5
0
*†
%PCNA
20
15
10
5
0
*†
□ Control
■ MCT
■ MCT+Ad-GFP
▨ MCT+Ad-GFP-S-M
B
MCT+Ad-GFP
DIC
Anti-BrdU + DAPI
50 μM
18x
MCT+Ad-GFP-S-M
DIC
Anti-BrdU + DAPI
50 μM
C
pA/pF
320
240
160
80
-80
-40
0
40
80
mV
*
MCT
MCT+Ad-GFP-S-M
MCT+Ad-GFP
% 4-AP inhibition
60
40
20
-80
-40
0
40
80
mV
*
MCT
Ad-GFP-S-M
2000 pA

Figure 3-8

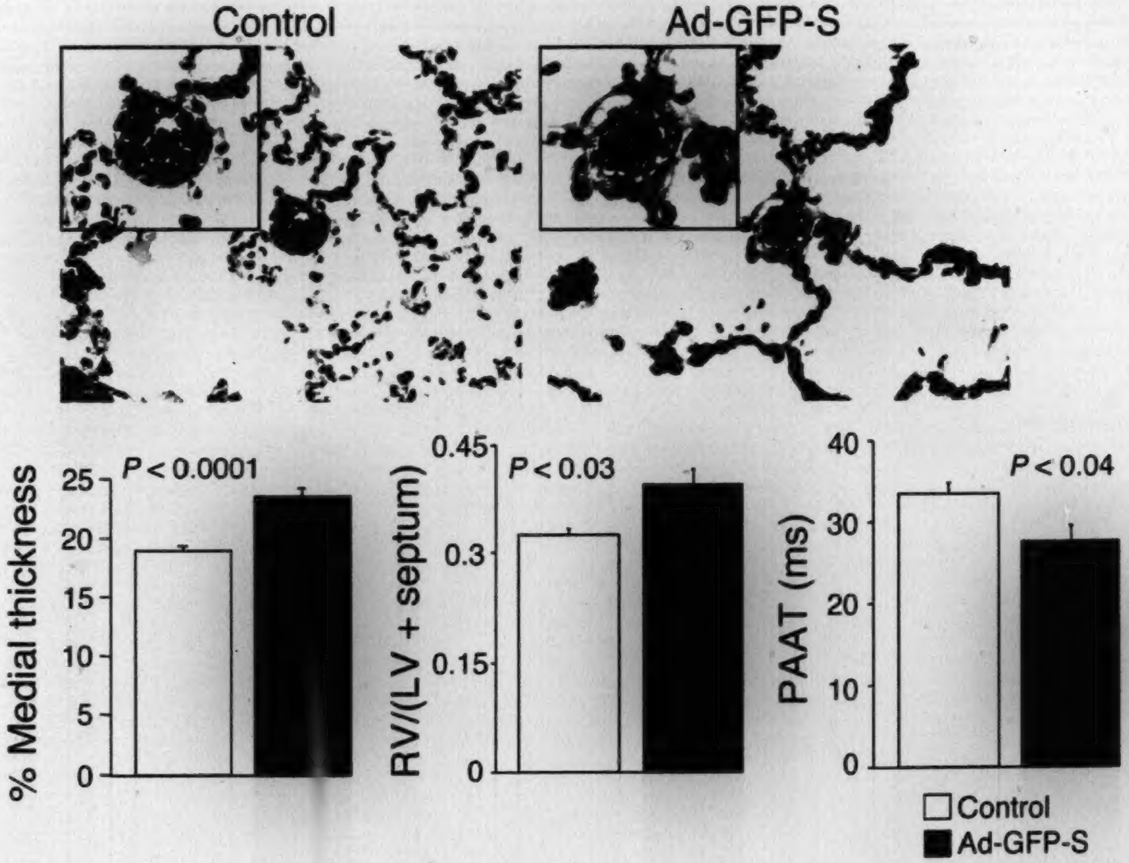
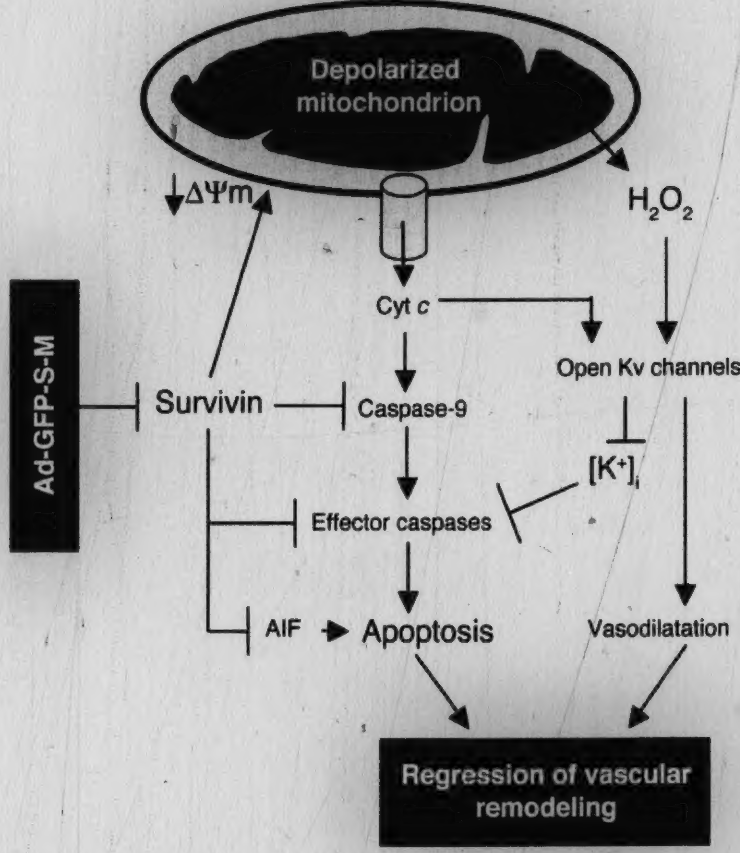

Control
Ad-GFP-S
% Medial thickness
25
20
15
10
5
0
$P < 0.0001$
RV/(LV + septum)
0.45
0.3
0.15
0
$P < 0.03$
PAAT (ms)
40
30
20
10
0
$P < 0.04$
□ Control
■ Ad-GFP-S

Figure 3-9


Depolarized mitochondrion
↓ΔΨm
H_2O_2
Ad-GFP-S-M
Survivin
Cyt *c*
Caspase-9
Open Kv channels
$[K^+]_i$
Effector caspases
AIF
Apoptosis
Vasodilatation
Regression of vascular remodeling

Combination therapy with rapamycin and atorvastatin does not reverse monocrotaline pulmonary arterial hypertension

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Fax (780) 407-6032
Email: sarcher@cha.ab.ca

Abstract

Background: Pulmonary arterial hypertension (PAH) is characterized by obstructive remodeling of resistance pulmonary arteries related to excessive cell proliferation and impaired apoptosis. Rapamycin, an antiproliferative agent, has been reported to prevent (but not reverse) monocrotaline PAH in rats. Similarly, simvastatin (2mg/kg/day) has been shown to reverse established monocrotaline-PAH by induction of apoptosis in neo-intimal smooth muscle cells. We hypothesized that rapamycin would reverse established monocrotaline-PAH, and that combination therapy with rapamycin and atorvastatin would be synergistic.

Methods: Adult male Sprague-Dawley rats were randomized to saline injection (n=6) or MCT (60mg/kg IP, n=36). MCT Rats were randomized to gavage with vehicle, rapamycin (2.5mg/kg/day), or rapamycin + atorvastatin (10mg/kg/day), beginning day 12 post-monocrotaline. Echocardiographic and hemodynamic endpoints were assessed on day 24.

Results: Despite the high doses of both agents, neither rapamycin nor combination therapy significantly reduced mean pulmonary arterial pressure, echocardiographic indices of pulmonary hypertension, remodeling of resistance PAs, or right ventricular hypertrophy. A weak trend for reduced pulmonary vascular resistance index was observed in both treatment groups. No synergy of the combination was observed. Rapamycin significantly attenuated phosphorylation of P70 S6 kinase, confirming adequacy of rapamycin dosing.

Conclusion: Neither rapamycin nor rapamycin plus atorvastatin reduced established MCT pulmonary hypertension. It is important to report the results of negative trials of experimental PAH therapy in rodents, particularly as these agents are being considered for human trials.

Role of M. S. McMurtry

Dr. McMurtry performed the animal experiments and hemodynamic studies, as well as the histology and RV remodeling experiments. He also performed immunohistochemistry. The immunoblots were performed by technicians with assistance from Dr. McMurtry. All data was collected, integrated and analyzed by Dr. McMurtry.

Introduction

Pulmonary arterial hypertension (PAH) is a syndrome characterized by proliferative and obstructive remodeling of the resistance pulmonary arteries (PAs)(12), leading to increased pulmonary vascular resistance (PVR) and right ventricular hypertrophy (RVH). Ultimately patients succumb to right heart failure and premature death(32). While drugs that are considered to be vasodilators have been the traditional mainstay of PAH therapy(7, 13), it is increasingly recognized that a cure for PAH will likely involve drugs, or drug combinations, that target the excess proliferation and disordered apoptosis that occurs within the resistance pulmonary arteries in PAH(21, 31). We examine two promising PAH therapies, rapamycin(26) and HMGCoA reductase inhibitors (statins)(27) in a rodent model of PAH induced by injection of monocrotaline, an alkaloid derived from Crotalaria spectabilis. An advantage of these drugs, should their benefit be confirmed, is that they are available in clinical practice.

Rapamycin is an immunosuppressant originally isolated from the bacterium *Streptomyces hygroscopicus*(36). Rapamycin binds to a intracellular receptor called FKBP12(1), and the rapamycin-FKBP12 complex binds mTOR (mammalian target of rapamycin) (2, 11, 33), a ~280 kDa serine/threonine kinase(6). This rapamycin-FKBP12-mTOR complex activates S6 kinase, which phosphorylates S6 (a 40S ribosomal protein) and in so doing modulates the translation of ribosomal proteins and translation elongation factors(35), arresting cells in the late G1 phase of the cell cycle(6). Rapamycin has clinical utility in transplant medicine as an immunosuppressant(15), in cardiovascular medicine as an antiproliferative agent to reduce in-stent restenosis(24), and has promise as an anti-cancer agent(28).

Recently, Nishimura et al reported that rapamycin attenuates PAH and suppresses neo-intimal proliferation in a model induced by the combination of pneumonectomy plus monocrotaline (MCT, 60mg/kg) (26), a variant of the monocrotaline model of PAH(4, 19, 20, 37). Interestingly, this study found that rapamycin, although it prevented PAH, it failed to reverse established PAH. This is an important limitation of this strategy if verified as true.

Simvastatin is a 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor ("statin") that is used widely in the primary and secondary prevention of atherothrombosis(8, 18). Simvastatin, as well other statins, are postulated to have beneficial "pleiotropic" effects on the vasculature beyond LDL reduction(3, 5), including augmentation of endothelial function, nitric oxide mediated vasodilation and inhibition of proliferation of endothelial and vascular smooth muscle cells. Simvastatin has been shown in one study to reverse established MCT PAH in rats by inducing apoptosis of neo-intimal smooth muscle cells(27). Although there are no randomized human trials of simvastatin in PAH, it is being used in some patients at some centers and a positive observational series has been published(16). Furthermore, there is a clinical trial, based at Imperial College London, currently randomizing patients with PAH to simvastatin or placebo (ClinicalTrials.gov Identifier: NCT00180713). Atorvastatin is a more potent statin than simvastatin, in terms of reducing cholesterol and cardiovascular endpoints(9), and may have more potent pleiotropic effects, such as enhancement of endothelial derived nitric oxide availability, suppression of inflammation, and inhibition of oxidative stress, as well(17).

We hypothesized that combination therapy with high dose rapamycin 2.5 mg/kg/day and atorvastatin 10mg/kg/day would have synergistic beneficial effects on the pulmonary vasculature, and would reverse established MCT PAH in rats.

Materials and Methods

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

Experimental Protocols. All experiments were conducted with ethical approval from the University of Alberta Animal Policy and Welfare Committee. Adult male Sprague-Dawley rats (200-300g) were used. Rats were injected with monocrotaline 60mg/kg ip or saline vehicle. Rats injected with monocrotaline were then randomized to placebo, rapamycin 2.5mg/kg/day, or rapamycin 2.5mg/kg/day plus atorvastatin 10mg/kg/day (n=12 per group), beginning on day 12 for 12 days prior to invasive evaluation. These high doses were based on the literature(25, 26) and used in order to avoid underdosing in case of negative results. Both rapamycin and atorvastatin were given by a single daily gavage, which ensured the medication was ingested. Rapamycin was purchased in oral liquid form (Wyeth Pharmaceuticals, Markham, Ontario). Atorvastatin (Pfizer, Kirkland, Quebec) tablets were crushed and suspended in simple syrup (2 parts sugar, 1 part distilled water) to facilitate gavage. Doses were freshly prepared each day. Echocardiography and invasive hemodynamic measurements (see below) were made on day 24 post monocrotaline, followed by animal sacrifice.

Echocardiography and Hemodynamics . RV free wall thickness and PA Doppler signals were measured in the parasternal short axis view at the level of the aortic valve using a Sonos 5500 echo machine with a 15-MHz probe (Phillips). Rats were anaesthetized with ketamine (60mg/kg ip) and xylazine (20mg/kg ip) and placed on a warmed surgical stage. Invasive left heart catheterization (carotid artery pressure and left ventricular pressure) and right heart catheterization (pulmonary artery pressure) were performed, as previously described, using a Millar catheter (Millar Instruments Inc., Houston, Texas)(22). Cardiac output was measured in triplicate by a validated thermodilution method using a thermistor probe (ADInstruments, Colorado Springs, Colorado) and 0.5ml injections of iced saline(29). PVRi was calculated as (mean PA – LVEDP)/cardiac index.

Morphometric analysis of RVs and PAs. RVH was measured as RV/(LV + septum) weight ratio at sacrifice. Lungs were inflated with formalin, fixed overnight, and embedded in paraffin. Tissue was stained with H&E or anti-vWF antibody. Five rats per group were studied, and from each rat at least 2 separate lung sections were examined. Resistance PAs (20–150 μm) chosen randomly from low-power fields were analyzed (approximately 50 arteries per group; 2–3 slides per rat) by 2 blinded investigators using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). External diameter (ED) and medial thickness (MT) were measured, and percent medial thickness was calculated as 2 × MT × 100/ED.

Immunoblots. Protein expression in whole lungs was measured with immunoblotting using available antibodies, as previously described(23). The actin and phospho-P70 S6 Kinase (Thr389) primary antibodies were rabbit and goat polyclonal antibodies respectively (Santa Cruz Biotechnology, Santa Cruz, CA). The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system (Kodak, Toronto, ON).

Statistics. Sample sizes were based on previous studies of rapamycin (n=5 per group) and simvastatin (n=12 per group) which demonstrated benefit. Assuming a treatment effect of a reduction of mean PAP of 10mmHg, the estimated power for a sample size of 6 per group was 89%. Values are expressed as the mean ± SEM. Kruskal-Wallis test or ANOVA was used as appropriate. Fisher's probable least-significant difference test was used for post hoc analysis, only if the overall ANOVA indicated significance (StatView 4.02; SAS Institute Inc.). A $p<0.05$ was considered statistically significant.

Results

Rats injected with monocrotaline developed PAH and RVH by day 10, which was severe by day 24. All rats survived to have echocardiography and invasive hemodynamic measurements. No significant reduction was observed in mean pulmonary arterial pressure with either rapamycin or rapamycin+atorvastatin therapy (Figure 1, Figure 2). Similarly, there were no differences in cardiac index or systemic blood pressure in the treatment groups versus placebo. No significant decrease in pulmonary vascular resistance index (PVRI) was observed with either therapy versus placebo, but there was a trend towards a small benefit in both the rapamycin (80.6±5.8 vs. 94.9±5.8 mmHg*min*kg/ml, p=0.07) and rapamycin+atorvastatin groups (80.3±5.8 vs. 94.9±5.8 mmHg*min*kg/ml, p=0.07) versus placebo (Figure 2). No synergy was observed with combination rapamycin+atorvastatin over rapamycin alone.

Echocardiographic measurements were in accordance with the invasive hemodynamics. No significant differences between the groups were observed for right ventricular thickness, pulmonary artery acceleration time (PAAT, a validated measure of mean PAP which shortens as mean PAP rises(14)), or right ventricular hypertophy as measured by the RV/LV+S ratio (Figure 3).

Monocrotaline increased the percent medial thickness of intra-parenchymal resistance PAs from 24.4±1.4% to 40.1±1.6% (Figure 4). None of the therapies reduced this vascular remodeling.

Phosphorylation of P70 S6 kinase in homogenized lung tissue was reduced by rapamycin treatment versus the monocrotaline group, confirming the adequacy of rapamycin doing (Figure 5). Despite the lack of hemodynamic effects of rapamycin, the elevation of phosphorylated P70 S6 kinase supports the rationale for this treatment strategy.

Discussion

This study carefully tested two promising oral therapies for PAH, using drugs that are available for patients and have previously been reported to be beneficial in experimental PAH. In contrast to the hypothesized benefit of synergy from the combination of rapamcyin and statin versus placebo, we show that neither rapamycin 2.5mg/kg/day nor combined rapamycin 2.5mg/kg/day and atorvastatin 10mg/kg/day significantly reversed established monocrotaline PAH. Though disappointing, our results actually confirm those of Nishimura et al who found that whereas rapamycin would prevent PAH if given at the same time as moncrotaline, it was ineffective in reversing established monocrotaline PAH(26). It is unlikely that higher doses or rapamycin would be useful, as 2.5mg/kg/day is a very high dose compared to doses currently used in patients (maintenance doses of 2mg/day in adults > 40kg), and higher doses are limited by toxicity. We chose not to test a prevention strategy as this is of limited relevance to PAH, a syndrome where presentation and diagnosis occurs almost inevitably relatively late in the course of the disease.

Unlike previous studies of simvastatin(27) with similar sample size, we did not show significant reversal of monocrotaline PAH with the rapamycin+atorvastatin group. No increased benefit was seen with the combination therapy over the rapamycin alone. This result is in stark contrast to the marked improvements in pulmonary hemodynamics seen with simvastatin, which reduced mean PAP from 42 to 36 mmHg at 2 weeks and to 24 mmHg at 6 weeks(27). It is unlikely that the atorvastatin was underdosed, as the simvastatin dose used in the previous study was 2mg/kg/day and in the present study atorvastatin was dosed at 10mg/kg/day. A shorter duration of therapy may possibly have contributed, as the duration of treatment in the simvastatin therapy study varied from 2 weeks to six weeks, compared with 12 days in our study. There may also be differences in the two models used. Although the MCT dose was 60mg/kg IP for each study, Nishimura et al. used pneumonectomized rats at sea level versus our non-pneumonectomized rats at 668m above sea level. Nishimura et al. achieved a mean PA pressure of approximately 40mmHg in the vehicle treated controls, versus our model which achieved a mean PA pressure of 34mmHg. Higher PA pressures to start with might offer a greater chance for demonstrating benefit. Additionally, the theoretical possibility exists that rapamycin somehow masked a potential beneficial effect of atorvastatin. This seems unlikely, however, given the known mechanisms of each drug and their frequent concomitant use in transplant patients.

Why would a drug such as rapamycin that prevents PAH not regress the established disease? There is biological plausibility for an antiproliferative strategy only working if given prior to the PAH-causing insult. Quinlan et al. found that PASMC proliferation was increased ~ 50% in hypoxic mice exposed at day 4-6 days; however, after 3 weeks of hypoxia, the proliferative index had returned to normoxic levels, despite the occurrence of pulmonary hypertension(30). Perhaps there is a limited window of time, early in PAH, when an antiproliferative strategy is beneficial.

Simvastatin and atorvastatin have different chemistries, which in turn cause different pharmacokinetics and pharmacodynamics and thus different potencies and pleiotropic effects(3, 17). Although atorvastatin is generally believed to be more potent than simvastatin in terms of LDL reduction and certain pleiotropic effects, including anti-inflammatory and anti-oxidant properties, it is possible, though in our opinion very unlikely, that simvastatin has more potent pleiotropic effects that are most relevant in monocrotaline PAH. For example, it has been suggested that simvastatin is a more potent coronary artery vasodilator than atorvastatin, perhaps due to the presence of a lactone ring(10). It might be that simvastatin behaves as a potent pulmonary vasodilator in monocrotaline PAH, as opposed to an anti-proliferative therapy, and this may account for the 100% survival benefit (versus control rats at 15 weeks post monocrotaline) observed by Nishimura et al(27).

Interestingly, there was a trend (p=0.08) towards a reduction of right ventricular hypertrophy with combination therapy of rapamycin and atorvastatin compared to the placebo treated rats. This was not observed in the rapamycin group, which suggests a possible beneficial effect of atorvastatin in reduction of right ventricular hypertrophy. This observation is consistent with published reports of reductions of left ventricular hypertrophy by atorvastatin in hypertensive rats(38) and a transgenic rabbit model of human hypertrophic cardiomyopathy(34). Whether a potential reduction in right ventricular hypertrophy without improvement in pulmonary hemodynamics would translate to a clinical benefit, such as improved mortality, improved functional capacity, or symptom reduction is unknown.

While our experiments did not support our hypothesis, these are important results to report. A search of PubMed reveals that since January of 2000 66 studies have demonstrated efficacy in the prevention or reversal of monocrotaline pulmonary hypertension in rodents, and no negative studies have been published. It is likely the literature is biased towards positive studies, with negative results being under-reported.

Since there is currently a clinical trial of simvastatin in human PAH ongoing, reporting negative trials of stain therapy in animal models of PAH is of paramount importance.

Conclusions

We conclude that rapamycin does not significantly reverse established MCT PAH, and therefore may be of limited benefit in human PAH. Similarly, combination rapamycin and atorvastatin does not reverse established monocrotaline PAH. Perhaps further study of the role of statins in human PAH should await the positive results derived from studies of regression of experimental PAH conducted by multiple investigators in multiple PAH models.

Acknowledgements

S.L. Archer is supported by the Canada Foundation for Innovation, the Alberta Heart and Stroke Foundation, the Alberta Heritage Foundation for Medical Research, the Canadian Institutes for Health Research, the Alberta Cardiovascular and Stroke Research Centre (ABACUS) and by NIH grant HL071115. S.L. Archer is Canada Research Chair in Translational Cardiovascular Medicine and Oxygen Sensing.

E.D. Michelakis is supported by the Canada Foundation for Innovation, the Alberta Heart and Stroke Foundation, the Alberta Heritage Foundation for Medical Research, the Canadian Institutes for Health Research, and the Alberta Cardiovascular and Stroke Research Centre (ABACUS). E.D. Michelakis is Canada Research Chair in Pulmonary Hypertension.

M.S. McMurtry is supported by the Clinician Investigator program at the University of Alberta and a training grant from Bristol-Myers Squibb Co.

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Figure Legends

Figure 4-1 – Representative traces of pulmonary artery pressure at cardiac catheterization (A). Parasternal short axis view demonstrating measurement of RV thickness (B) and PA doppler flow interrogation (C).

Figure 4-2 – There is no significant difference between either rapamycin or rapamycin+atorvastatin versus placebo in terms of mean pulmonary arterial pressure, systemic blood pressure, or cardiac index. Similary, there was no significant difference between the groups in terms of pulmonary vascular resistance index, although there was a trend toward a modest benefit compared to placebo with both treatment arms.

Figure 4-3 – Echocardiographic indices of PAH, including right ventricular thickness and pulmonary artery acceleration time, as well as right ventricular weight divided by the left ventricle plus septum weights, were not significantly different in the treated groups versus the placebo group.

Figure 4-4 – PAH was associated with increased resistance PA remodeling (%medial thickness), but this was not reduced by either rapamycin or rapamycin+atorvastatin therapy.

Figure 4-5 – Rapamycin treatment reduces phosphorylation of P70 S6 kinase in homogenenized lung tissue, consistent with adequate rapamycin dosing. MCT increases

the phosphorylation of P70 S6 kinase, confirming the rationale for the study.

Figure 4-1

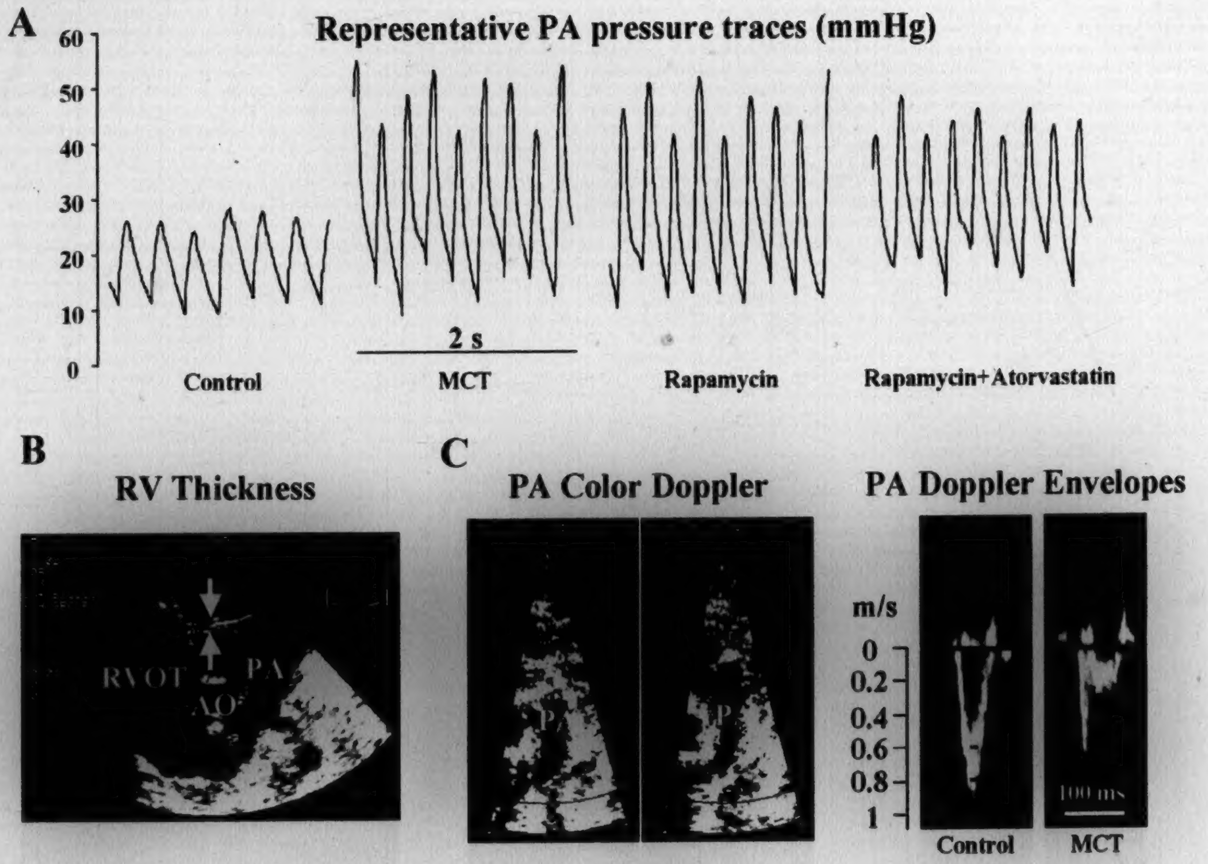

A
Representative PA pressure traces (mmHg)
60
50
40
30
20
10
0
2 s
Control
MCT
Rapamycin
Rapamycin+Atorvastatin
B
RV Thickness
RVOT
PA
AO
C
PA Color Doppler
P
P
PA Doppler Envelopes
m/s
0
0.2
0.4
0.6
0.8
1
100 ms
Control
MCT

Figure 4-2 **Invasive Hemodynamics**

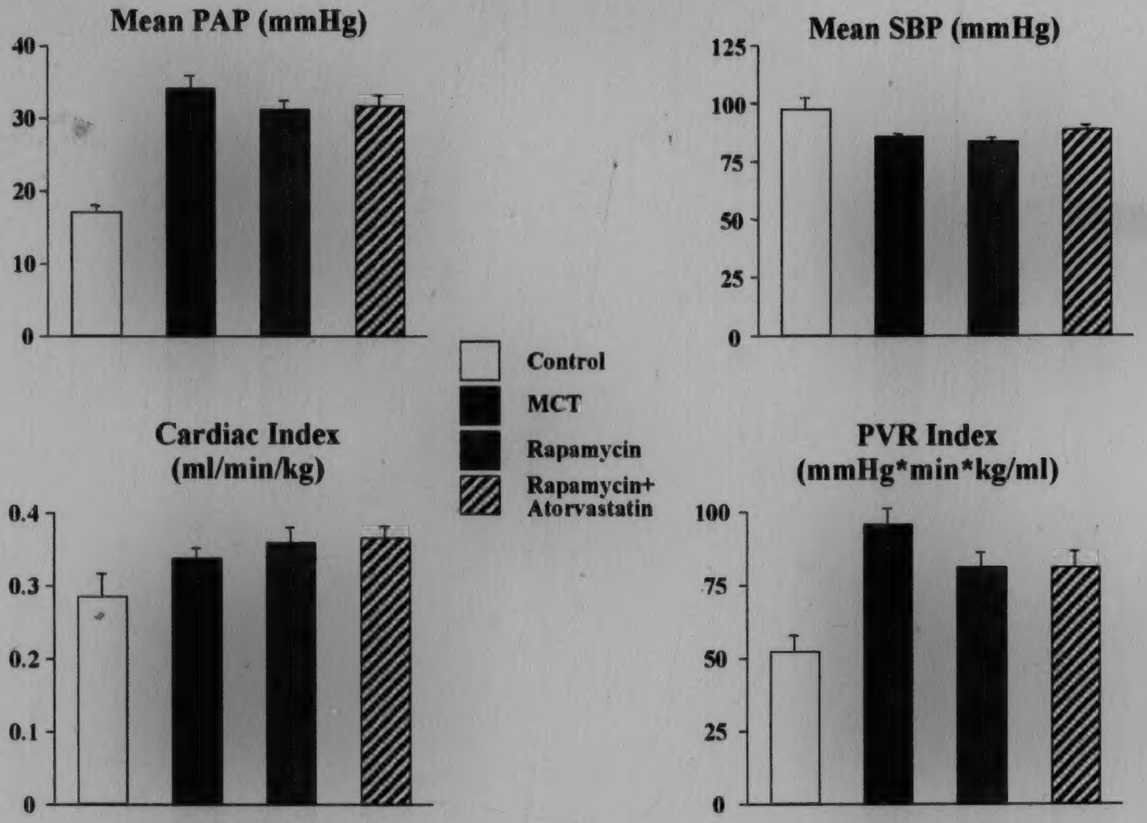


Figure 4-3 Echo Parameters of PAH

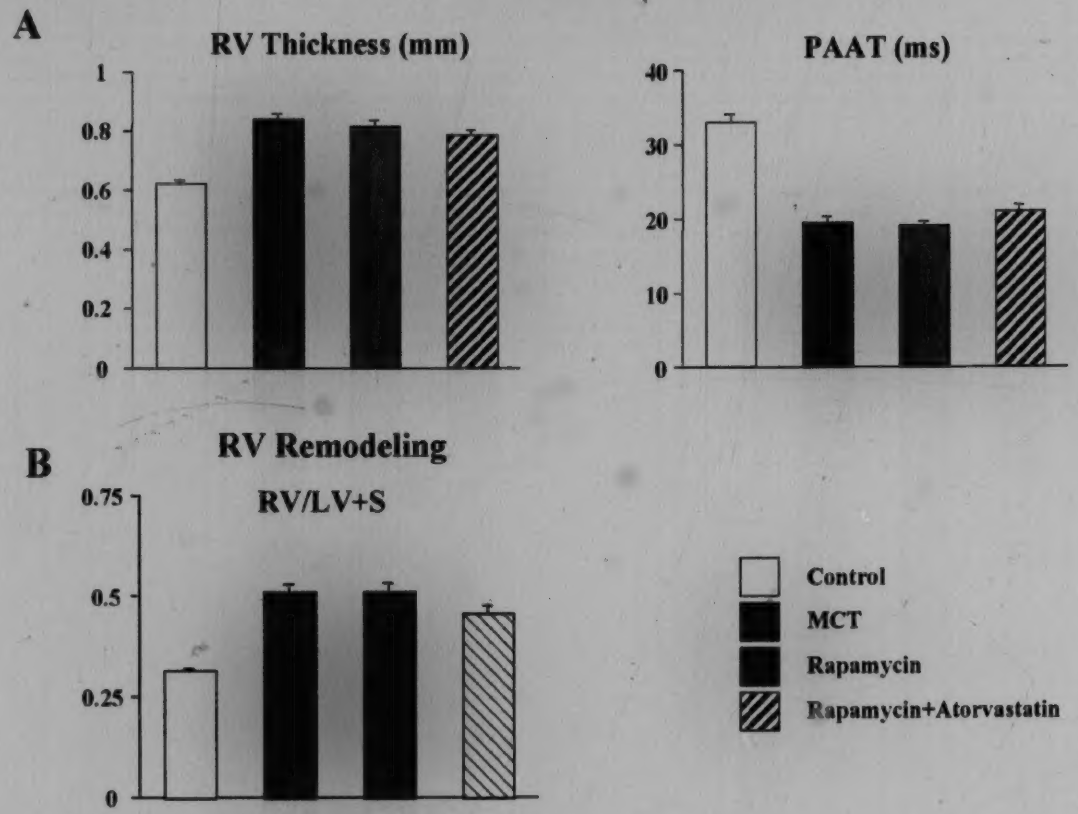


Figure 4-4 **Resistance PA Remodeling**

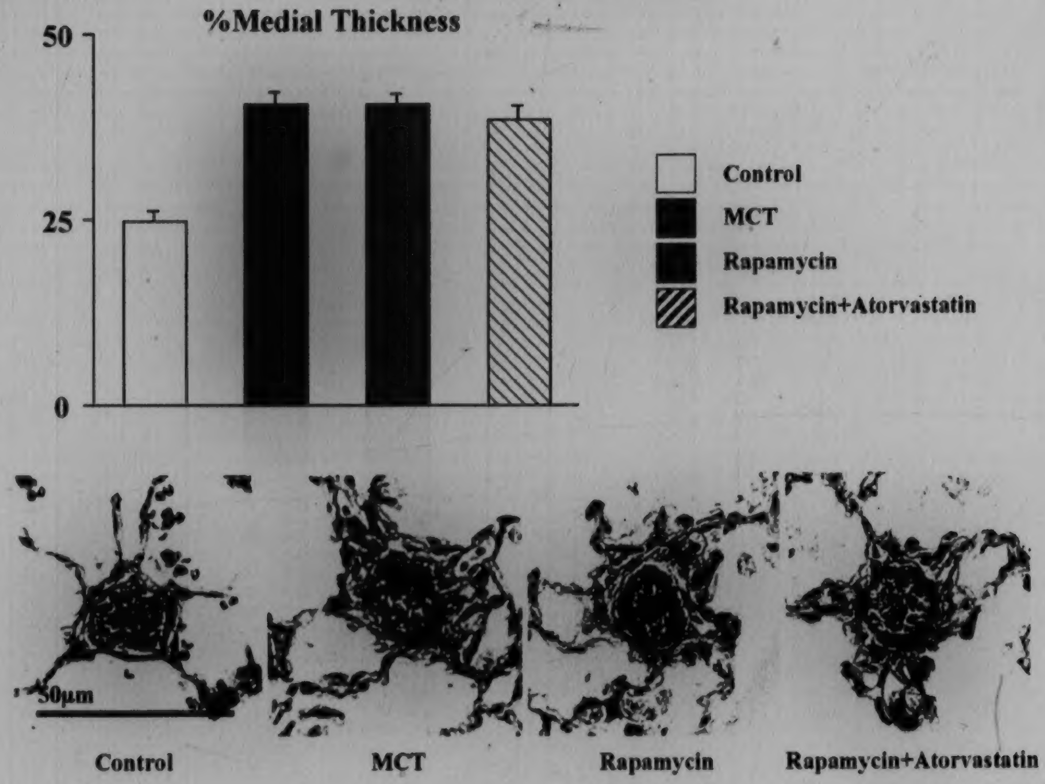
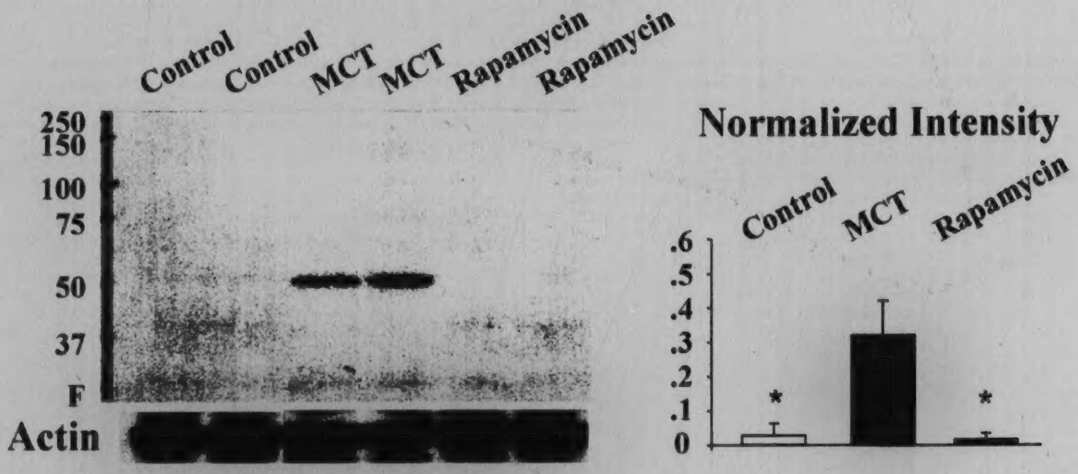


Figure 4-5

Anti-phospho-P70 S6 kinase



Overexpression of human bone morphogenetic protein receptor II does not ameliorate monocrotaline pulmonary arterial hypertension

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Role of M. S. McMurtry

Dr. McMurtry performed the animal experiments and hemodynamic studies, as well as the histology and RV remodeling experiments. He performed the qRT-PCR, LCM, and immunohistochemistry. The immunoblots, confocal microscopy and electrophysiology experiments were performed by technicians with assistance from Dr. McMurtry. All data was collected, integrated and analyzed by Dr. McMurtry.

Abstract

Background: Pulmonary arterial hypertension (PAH) is associated with mutations of bone morphogenetic protein receptor II (BMPR2) and BMPR2 expression decreases with the development of experimental PAH. Decreased BMPR2 expression and impaired intracellular BMP signaling in PA smooth muscle cells (PASMC) suppresses apoptosis and promotes proliferation thereby contributing to the pathogenesis of PAH. We hypothesized that overexpression of BMPR2 in resistance PAs would ameliorate established monocrotaline PAH.

Methods: Human BMPR2 was inserted into a serotype 5 adenovirus with a green fluorescent protein reporter (GFP). Dose-dependent, transgene expression was confirmed in PASMC cells using fluorescence microscopy, qRT-PCR and immunoblots. PAH was induced by injecting Sprague Dawley rats with monocrotaline (60mg/kg ip) or saline. On day 14 post MCT monocrotaline rats received $5X10^9$ pfu of either Ad-hBMPR2 or Ad-GFP. Transgene expression was confirmed by fluorescence microscopy, quantitative RT-PCR of whole lung samples, and laser capture micro-dissected resistance PAs. Invasive hemodynamic and echocardiographic endpoints of pulmonary hypertension were assessed on day 24.

Results: Endogenous BMPR2 mRNA levels were greatest in resistance PAs and expression declined with MCT-PAH. Despite robust hBMPR2 expression in all lung lobes and within resistance PAs of treated rats, hBMPR2 did not lower mean PA pressure, pulmonary vascular resistance index, RV hypertrophy, or remodeling of resistance PAs.

Conclusion: Nebulized intratracheal adenoviral gene therapy with hBMPR2 reliably distributed hBMPR2 to resistance PAs, but did not ameliorate PAH. Depressed BMPR2 expression may be a marker of PAH but is not central to the pathogenesis of this model of PAH.

Introduction

Pulmonary arterial hypertension (PAH) is a syndrome characterized by proliferative remodeling and obliteration of resistance pulmonary arteries (PAs). The high pulmonary vascular resistance (PVR) eventually causes failure of the afterload-intolerant right ventricle and, despite advances in therapy, PAH continues to result in high mortality rates (6, 26), with a recent estimate of 88% survival at 1 year in a French cohort of 674 patients(16). Despite the fact that only 20% of PAH patients have a significant vasoconstrictor component to their pulmonary hypertension, current therapy for PAH is largely limited to agents selected for their vasodilator properties, although it is now clear that some of these agents also have antiproliferative effects(11, 35). PAH therapies include prostacyclin and prostacyclin analogues(5), endothelin receptor blockers(9), and phosphodiesterase 5 inhibitors(14) and calcium channel blockers(2). Recently it has been appreciated that PAH is predominantly a disease of excess cell proliferation and impaired apoptosis(36), raising the possibility of therapies that target the proliferative remodeling of the resistance PAs, such as dichloroacetate(21), simvastatin(32), anti-survivin(20) or K+ channel replacement therapy(27).

A genetic cause for PAH had long been suspected based on the occurrence of familial PAH. A recent study indicates that in France familial PAH accounts for 3.9% of all PAH(16). Familial PAH relates to mutations of bone morphogenetic protein receptor type-2 (BMPR2) and activin-like kinase type-1 (ALK-1) receptors(25). Mutations of BMPR2 occur in ~75% of patients with familial PAH(12, 17); however they appear to be relatively rare in sporadic PAH (~10%) (25). A member of the TGF-β superfamily, BMPR2 is crucial to embryologic growth and development(8). BMPR2 is a constitutively active serine-threonine kinase receptor which, in response to ligand binding (BMP2, 4, 7), forms hetero-dimers with any of 3 type 1 receptors (BMPR1A, BMPR1B or ALK). This association results in phosphorylation of the intracellular portion of the type 1 receptor by BMPR2, initiating a cytosolic protein signaling cascade consisting of Smad proteins (30). Receptor activated (R-SMADs), including Smad 1, 5 and 8, complex with a common partner SMAD (Smad4) permitting it to translocate to the nucleus where it can regulate gene transcription. The Smad DNA interaction is weak and requires co-repressors or activators. In the nucleus, R-Smad/co-Smad complexes interact with genes that have a Smad-binding element (5-AGAC-3), altering PASMC proliferation and apoptosis. BMPR2 receptors are expressed not only in human endothelium but also in human PASMCs, where their activation by BMPs normally leads to phosphorylation of SMAD1, decreased Bcl expression and induction of apoptosis(37). This antiproliferative effect of BMPs, which is partially due to induction of PASMC apoptosis, is suppressed in PAH(37).

In support of the BMPR2-hypothesis of PAH, reduced pulmonary vascular BMPR2 expression has been described in human PAH(4), and PASMCs from patients with PAH proliferate abnormally (excessively) in response to BMP ligands(24). Mice with haploinsufficiency of BMPR2 have mild PAH(7, 34), and may be more susceptible to PAH when exposed to pulmonary vascular stressors(18, 31). Discoveries of related TGF-β superfamily members that are associated with PAH and hereditary hemorrhagic telangiectasia, such as ALK 1(33) and endoglin(10), have strengthened the case for BMPR2 playing an important role in the development of PAH. Interestingly, abnormalities on the TGF-BMP pathways have been described in tumors, like the juvenile colonic polyposis(29), or vascular lesions such as the coronary restenosis lesions post angioplasty(19). Monocrotaline PAH, an animal model of PAH, is relatively irreversible, usually fatal, and recapitulates many features of human PAH. Based on the evidence implicating deficient BMPR2 in the development of PAH, we hypothesized that overexpression of BMPR2 in the pulmonary vascular tree using an adenoviral gene therapy approach would ameliorate or reverse established monocrotaline PAH in the rat.

Materials and Methods

Experimental Protocols. All experiments were conducted with ethical approval from the University of Alberta Animal Policy and Welfare Committee. Adult male Sprague Dawley rats (200-300g) were used. Rats were injected with monocrotaline (MCT) 60mg/kg ip or saline (vehicle), and 14 days later were randomized to receive either nebulized adenovirus coding for human BMPR2 (Ad-hBMPR2) or green fluorescent protein (Ad-GFP) each at 5X10^9 pfu, n=17 rats per group. This airway gene therapy was delivered to anaesthetized, intubated rats via a nebulizer needle (Microsprayer, Penn Century Inc., Philadelphia, PA), as previously described(27). Echocardiography and invasive hemodynamic measurements were performed 24 days following monocrotaline injection (~10 days after inhalational therapy) and the rat was then sacrificed.

Human BRMPII adenovirus. A replication deficient adenovirus encoding BMPR2 and/or green fluorescent protein (GFP) was generated as previously described(27) (Figure 1). Briefly, sense and anti-sense primers of BMPR2 carrying a myc tag were synthesized and polymerase chain reaction (PCR) was performed on a cDNA template (synthesized by reverse transcriptase PCR from mRNA obtained from a donor pulmonary artery during cardiac transplantation). The BMPR2-myc was propagated with TA Cloning kit (Invitrogen, Burlington, ON) and was subsequently inserted into a pAdTrack-CMV. The resultant pAdTrack-CMV-BMPR2myc construct was linearized with a PmeI restriction endonuclease digest and transformed, together with an adenoviral plasmid (Adeasy-1, Stratagene, La Jolla, CA), into bacterial BJ5183 cells (ATCC, Manassas, VA) which were plated on LB agar containing kanamycin. The selected colonies containing BMPR2-myc were isolated, amplified, purified and linearized (PacI endonuclease digest) and transfected into HEK 293 cells (ATCC, Manassas, VA) using LipofectAmine reagent (Invitrogen, Burlington, ON). Plates with complete cell lysis were collected and analyzed for BMPR2 expression with PCR and western immunoblots. Purification of the adenovirus was done by step-wise, discontinuous CsCl gradient. The viral titre was determined by measuring plaque forming units (PFU) per ml using a plaque assay involving HEK 293 cells in DMEM growth medium solution studied in 6-well plates.

Cell Culture. Isolated PAs (fourth to fifth division) were mechanically denuded of endothelium and digested by papain (1 mg/ml), DTT (0.5 mg/ml), collagenase (0.6 mg/ml), and bovine serum albumin (0.6 mg/ml; all from Sigma-Aldrich, Oakville, ON) for 20 minutes at 37°C. Cells were placed in culture medium supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% antibiotic/antimycotic (Invitrogen, Burlington, ON) and grown on 60mm plates for 3 days at 37°C. PASMCs were exposed to 100μl of 5×10^9 PFU/ml Ad-BMPR2 or Ad-GFP for 6 hours. Then cells were washed free of virus and after 48 hours in10% fetal bovine serum studied for transgene expression, using fluorescence microscopy and immunoblots.

Immunoblots. Protein expression in whole lungs was measured with immunoblotting using available antibodies, as previously described(23). The intensity of the bands was normalized to the intensity of a reporter protein (actin) using the Kodak Gel-doc system (Kodak, Toronto, ON).

Confocal Microscopy. Fluorescence imaging of either plates of PASMCs or frozen sections of whole lung tissue embedded in OCT and flash frozen was performed using a Zeiss LSM 510 confocal microscope.

Echocardiography and Hemodynamics. RV free wall thickness and PA Doppler signals were measured in the parasternal short axis view at the level of the aortic valve using a Sonos 5500 echo machine with a 15-MHz probe (Phillips, Markham, ON) under conditions of light anesthesia, as previously described(20, 21),. For invasive studies, rats were anaesthetized with ketamine (60mg/kg ip) and xylazine (20mg/kg ip) and placed on a warmed surgical stage. Invasive left heart catheterization (carotid artery pressure and left ventricular pressure) and right heart catheterization (pulmonary artery pressure) were performed as previously described using a micromanometer-tipped catheter (Millar Instruments Inc., Houston, TX)(21, 27). Cardiac output was measured in triplicate by a validated thermodilution method using a thermistor probe (ADInstruments, Colorado Springs, CO) and 0.5ml injections of iced saline. PVR index (PVRi) was calculated as (mean PA – LVEDP)/cardiac index.

Laser Capture Microdissection. Lungs were inflated with and embedded in OCT, flash-frozen, and cut in 10-μm sections using a Leica CM 1850 cryostat (Leica Microsystems Inc., Richmond Hill, ON). HistoGene slides and dehydration/staining reagents were used as previously described(1, 3). Laser-capture microdissection was performed using the PixCell II system (Arcturus, Mountain View, CA). The cap containing the excised material (3 resistance PAs per cap, 5 caps per group) was then transferred to the ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA) for measurement of mRNA using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

qRT-PCR. RNA extraction was performed using RNeasy (Qiagen, Mississauga, ON) and picopure extraction kits (Arcturus, Mountain View, CA). Samples were added to a microwell plate, along with TaqMan probes and reagents, and quantitative RT-PCR was performed, as previously described(21, 27). Except for 18S, all probes, including species-specific rat and human BMPR2 probes and housekeeping genes (SM22-α and 18S), were custom designed using Primer Express software and purchased from Applied Biosystems (Foster City, CA). mRNA abundance, relative to a housekeeping gene and the expression in other samples was expressed as $2^{\Delta\Delta Ct}$, as previously described(27).

Drugs and Statistics. Drugs were obtained from Sigma Aldrich (Oakville, ON) unless otherwise specified. Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Values are expressed as the mean ± SEM. Kruskal-Wallis test or single measure ANOVA was used, as appropriate. Fisher's probable least-significant difference test was used for post hoc analysis (StatView 4.02; SAS Institute Inc., Cary, NC). A $p<0.05$ was considered statistically significant.

Results

Heterologous expression of hBMPR2. Rat PASMCs incubated with Ad-hBMPR2 (and Ad-GFP-image not shown) were intensely fluorescent compared to controls, indicating efficient infection in vitro (Figure 2a). qRT-PCR confirmed dose-dependent transgene expression (Figure 2b). Immunoblots of protein extracted from the infected PASMCs cells showed robust expression of GFP and hBMPR2 protein, which were absent in control PASMC (Figure 2c).

Laser capture microdissection. Quantitative RT-PCR of laser capture microdissected pulmonary arteries (15 per group) from the proximal pulmonary arteries (extra pulmonary), mid pulmonary artery (airway associated), and distal pulmonary arteries (intraparenchymal) demonstrated a gradient of rat BMPR2 mRNA expression, with the distal pulmonary arteries having the highest expression levels. SM22α, a smooth muscle specific reporter, was used to account for varying levels of muscularization of these arteries (Figure 2d). The levels of endogenous rat BMPR2 expression in the distal (airway-associated) PAs were reduced in the rats treated with MCT, including both the GFP and hBMPR2 treated rats (Figure 2e). This also illustrates the species-specificity of the BMPR2 primers,

Gene therapy. Nebulized gene therapy with Ad-hBMPR2or Ad-GFP delivered transgene to the regions of the intraparenchymal resistance PAs, as shown by fluorescence confocal microscopy (Figure3a). qRT-PCR demonstrated robust and similar expression of hBMPR2 in all major lung lobes (Figure 3b). hBMPR2 was expressed in the resistance PAs collected by laser capture microdissection (Figure 3c).

Invasive hemodynamic measurements. There were no deaths in the MCT and MCT+hBMPR2 and MCT-GFP groups. There was no significant difference between rats treated with Ad-GFP, Ad-hBMPR2 and control moncortaline-treated rats in mean pulmonary artery pressure, mean systemic blood pressure, cardiac index, or pulmonary vascular resistance index (Figure 4). In accordance with the absence of hemodynamic improvement, there was no difference between the Ad-GFP rats and the Ad-hBMPR2 rats in echocardiographic indices of pulmonary hypertension, including pulmonary artery acceleration time (PAAT), a measure of mean PA pressure, and RV free wall thickness (Figure 5). Furthermore, Ad-hBMPR2 treatment did not reduce the hypertrophy of the right ventricle and distal resistance pulmonary arteries caused by monocrotaline (Figure 5). Upon qualitative inspection, no marked intimal thickening or fibrosis was observed in either control or treated animals. Immunoblots of whole lung tissue confirmed that BMPR2 protein was overexpressed in Ad-hBMPR2-treated rats in vivo (Figure 6).

Discussion

We report the first attempt to restore BMPR2 expression in experimental PAH. Although the transgene was effectively delivered, via airway nebulization, this did not ameliorate monocrotaline PAH. In addition to documenting the feasibility of augmenting receptor expression in vivo via an airway nebulization strategy, this work also demonstrates that BMPR2 expression in highest in the resistance pulmonary arteries. Moreover, we show that BMPR2 expression in small resistance PAs is reduced in experimental PAH (Figure 2d). This association supports a possible role for reduced BMPR2 expression and function in the development of both MCT PAH and human PAH. However, effective rescue of BMPR2, using gene therapy, was not beneficial.

Certain "malignant" BMPR2 mutations may be sufficient to cause PAH, with minimal requirement for a "second hit". Recently it was shown that SMC-specific overexpression of a BMPR2 mutant from family with a particularly malignant mutant results in PAH in transgenic mice(34). However, our results appear to indicate a lack of a causal role for BMPR2 deficiency in monocrotaline PAH. These findings are consistent with the evolving notion that PAH usually occurs as a result of multifactorial insults, i.e. that a BMPR2 mutation or deficiency, while permissive, is not usually sufficient to produce PAH. Although some BMPR2 haploinsufficient mice do develop mild pulmonary hypertension(7, 34), other such mice do not(18). A recent report found that mice with BMPR2 haploinsufficiency had normal pulmonary hemodynamics and vascular structure(18). Even when challenged with chronic hypoxia they did not develop more severe pulmonary hypertension than wildtype controls(18). However, these mice did manifest an exaggerated pulmonary hypertensive response to serotonin. Thus mice with

BMPR2 haploinsufficiency (at least some strains) are more susceptible to PAH(18, 31), even though they do not spontaneously develop severe PAH.

The lack of therapeutic efficacy of receptor replacement therapy is also concordant with the literature showing the limited penetrance (15-20%) of BMPR2 mutations in most PAH families (25, 28). Indeed, although most mutations are thought to result in loss of BMPR2 function, it is estimated that the lifetime risk of developing PAH for an unaffected BMPR2 mutation carrier (in an affected family) is only 10%(25).

Are there other reasons why BMPR2 gene therapy was not therapeutically beneficial? An otherwise efficacious therapy might fail because it did not reach its intended target tissue, or did not reach the target in an adequate dose. However, we show that the hBMPR2 transgene was expressed in the distal resistance PAs throughout the lungs, a finding that is particularly robust as transgene expression was confirmed in isolated resistance PAs harvested by laser capture microdissection. Underdosing of transgene is unlikely to have accounted for our negative therapeutic result, because the expression of hBMPR in the treatment group was similar to levels of rBMPR2 in controls. Moreover, net BMPR2 expression was increased in the treatment group relative levels seen in the control group (Figure 6). We do not believe that the results of this study can be attributed to the experiment being underpowered, as there was no trend for benefit with the Ad-hBMPR2 treatment, even using very sensitive surrogate end points such as % medial thickness of resistance PAs.

While under-dosing is unlikely to have accounted for our negative findings (based on the strong overexpression of human BMPR2 in the treatment group), it is possible that studying the animals only 10 days after the hBMPR2 therapy allowed insufficient time for beneficial changes to occur in the pulmonary vasculature. The choice of this time point reflected a compromise between the high spontaneous mortality that occurs in MCT-PAH after 4 weeks and the waning effect of adenoviral gene therapy after 14 days. A strength of our study is that we demonstrate that downregulation of endogenous BMPR2 expression occurs rapidly in MCT-PAH (within 3 weeks). This not only provided the rationale for replacement therapy but suggested that, if rapid downregulation of BMPR2 were causal, relatively brief replacement (10 days) should have been beneficial. The current study cannot exclude the possibility that benefit from BMPR2 gene therapy might have resulted from a different experimental design (e.g. of multiple rounds of therapy over a longer study time or prophylactic therapy). We elected not to do a prevention study because so many are positive and yet the opportunity to intervene before or concomitant with PAH does not occur clinically. An informal survey of PubMed reveals that there are approximately 5 times as many studies demonstrating prevention of PAH in an experimental model as showing any reversal of established disease over the last 2 years. Most PAH patients are 75% NYHA functional III upon presentation and have been symptomatic for several years(16). For these reasons there is a move away from prevention studies in PAH research. This decision to address the more rigorous standard (regression), also minimizes the number of animals sacrificed.

The relative contribution of abnormal BMP signaling in endothelium versus smooth muscle cells to the development of PAH is currently unknown(22). If the more important component of dysregulated BMP signaling occurs in the endothelium, it is possible that the therapy did not reach the necessary target. Further study is necessary to clarify this issue. The recent observation that BMP signaling in PA endothelial cells inhibits apoptosis (as opposed to inducing apoptosis in PASMC) is intriguing and it is in theory possible that if our transgene reached the endothelium (the virus reaches the PAs from the alveolar/adventitial, not the luminal, surface) its endothelial effects might have counteracted the effects on the PASMC.

An additional possible reason that monogene hBMPR2 therapy did not ameliorate monocrotaline-induced PAH is that other members of the BMP signaling cascade might also be abnormally expressed. Studies of human tissue comparing PAH patients with controls demonstrate hundreds of genes that are differentially expressed(13). If the distal effectors of the BMP signaling pathway are also reduced, such as SMADs or transcription factors(15), correcting the proximal BMPR2 deficiency may not be sufficient. Finally, we acknowledge that the pathology and pathogenesis of monocrotaline PAH is not identical to human PAH and that the failure of out hMBPR2 therapy might be due to other model-specific factors.

Conclusion

We conclude that successful replacement of BMPR2, achieved using an aerosolized gene therapy approach, does not ameliorate monocrotaline induced PAH. While this study does not exclude an important role for BMPR2 in the development of both animal and human PAH, it does suggest that BMPR2 deficiency by itself is not the central or most important molecular mechanism in this model.

Acknowledgements

S.L. Archer is supported by the Canada Foundation for Innovation, the Alberta Heart and Stroke Foundation, the Alberta Heritage Foundation for Medical Research, the Canadian Institutes for Health Research, the Alberta Cardiovascular and Stroke Research Centre (ABACUS) and by NIH grant HL071115. He holds a Canada Research Chair in Oxygen Sensing and Translational Cardiovascular Research.

E.D. Michelakis is supported by the Canada Foundation for Innovation, the Alberta Heart and Stroke Foundation, the Alberta Heritage Foundation for Medical Research, the Canadian Institutes for Health Research, and the Alberta Cardiovascular and Stroke Research Centre (ABACUS). E.D. Michelakis is Canada Research Chair in Pulmonary Hypertension.

M.S. McMurtry is supported by TORCH research training program and the Clinician Investigator program at the University of Alberta and a training grant from Bristol-Myers Squibb Co.

R. Moudgil is supported by training grants from CIHR, AHFMR and TORCH.

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Figure Legends

Figure 5-1 – Schematic diagram illustrating the generation of the Ad-hBMPR virus with GFP reporter both driven by CMV promoters.

Figure 5-2 – PASMCs in culture are efficiently transfected using the adenovirus construct and fluoresce due to expression of the green fluorescent protein (GFP, Figure 2a). Quantitative RT-PCR and immunoblots confirm dose dependent expression of GFP and BMPR2 (Figure 2b and 2c). Rat BMPR2 is predominantly expressed in the resistance as opposed to extra or intra-parenchymal conduit pulmonary arteries (Figure 2d), and Rat BMPR2 expression is decreased in MCT PAH (Figure 2e). The * indicates $p < 0.05$.

Figure 5-3 – Lung tissue exposed to adenovirus demonstrates green fluorescence in the neighbourhood of resistance PAs, suggesting effective transgene delivery (Figure 3a). Robust transgene expression was observed in all major lung lobes (Figure 3b). Laser capture microdissected resistance PAs robustly express the human BMPR2 transgene, confirming successful gene therapy delivery (Figure 3c). The * indicates $p < 0.05$.

Figure 5-4 – Pulmonary artery pressure as well as pulmonary vascular resistance index (PVRi) are not significantly improved by treatment with the human BMPR2 gene therapy. Similarly, there was no difference in mean systemic blood pressure or cardiac index.

Figure 5-5 – Echocardiographic indices of PAH, including PAAT and RV thickness, are not improved by treatment with the human BMPR2 gene therapy. Similarly, there was no significant improvement in right ventricular hypertrophy as measured by the RV/LV+S weight ratio, or percent medial thickness.

Figure 5-6 – Immunoblotting of whole lung tissue shows that hBMPR2 protein was expressed in target lung tissues.

Figure 5-1 **Schematic of Adenovirus endoding hBMPR2 under a CMV promoter**

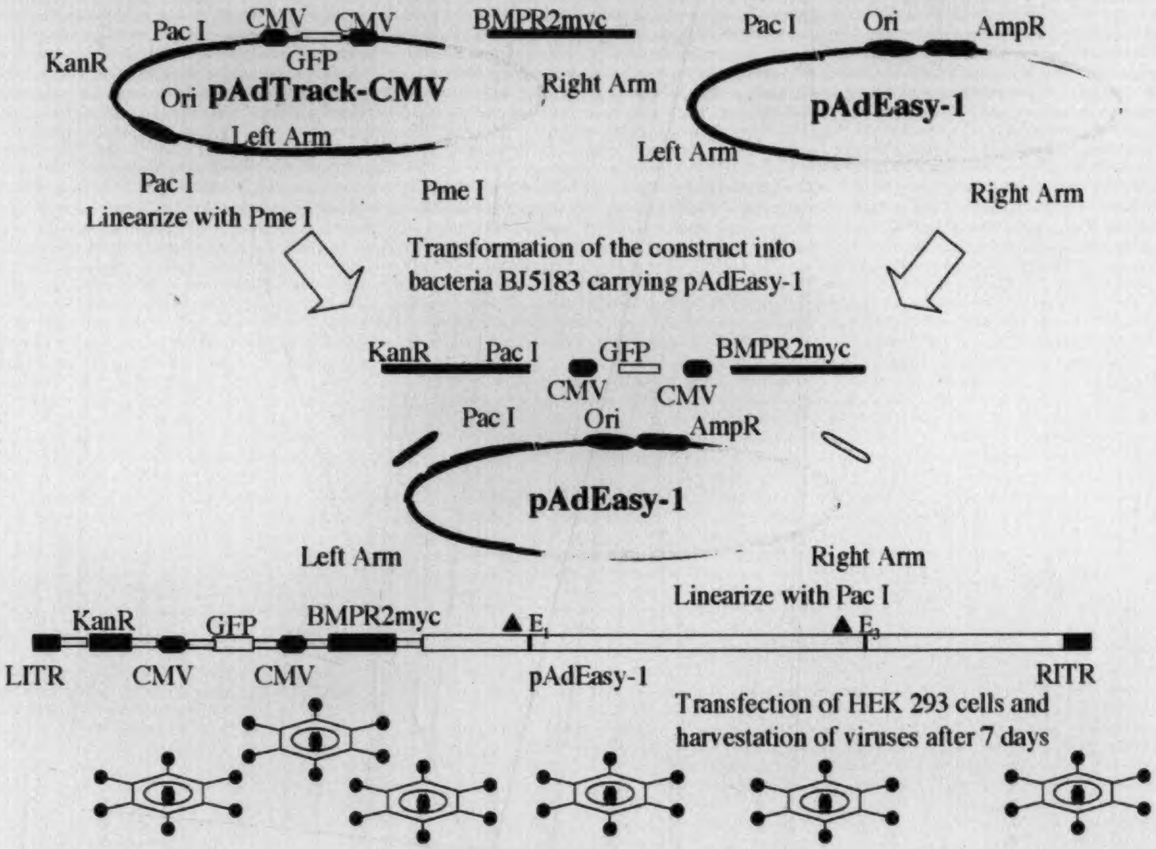

Pac I
CMV
CMV
KanR
GFP
Ori
pAdTrack-CMV
Left Arm
Pac I
BMPR2myc
Right Arm
Pme I
Linearize with Pme I
Pac I
Ori
AmpR
pAdEasy-1
Left Arm
Right Arm
Transformation of the construct into
bacteria BJ5183 carrying pAdEasy-1
KanR
Pac I
GFP
BMPR2myc
CMV
CMV
Pac I
Ori
AmpR
pAdEasy-1
Left Arm
Right Arm
Linearize with Pac I
KanR
GFP
BMPR2myc
E1
E3
LITR
CMV
CMV
pAdEasy-1
RITR
Transfection of HEK 293 cells and
harvestation of viruses after 7 days

Figure 5-2

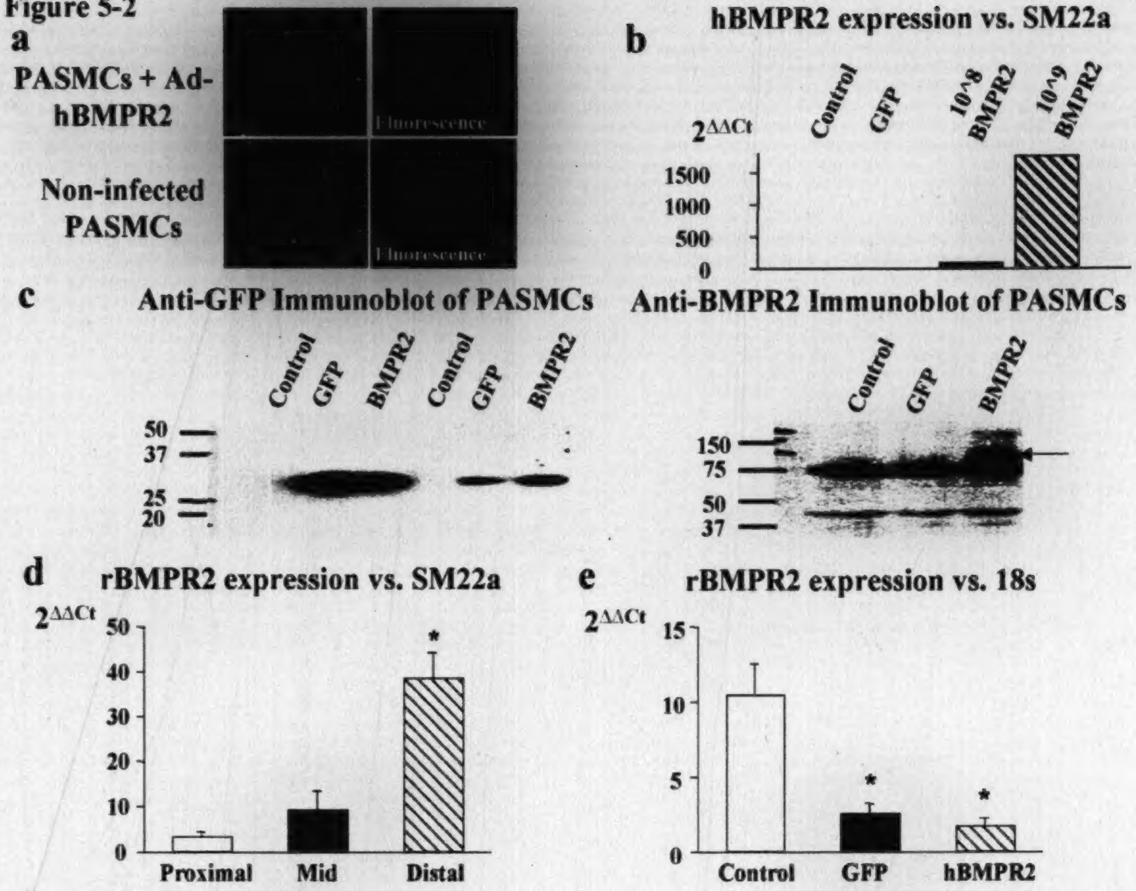
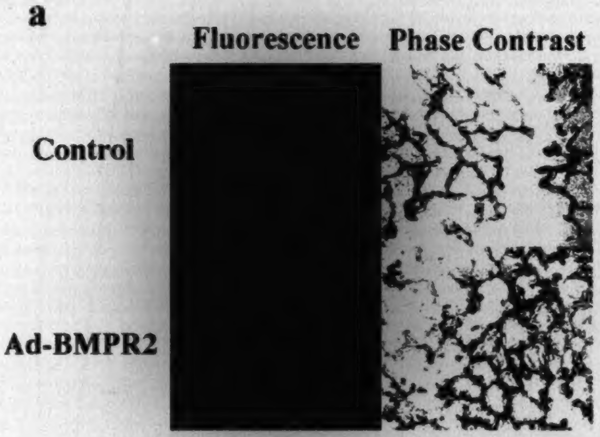
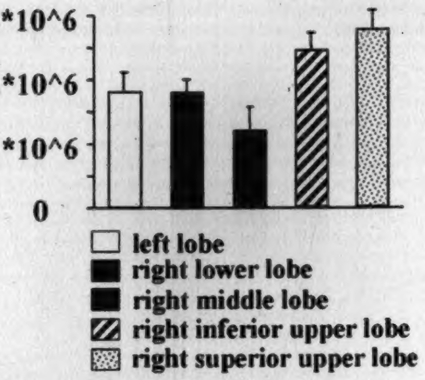

a
PASMCs + Ad-hBMPR2
Non-infected PASMCs
Fluorescence
Fluorescence
b
hBMPR2 expression vs. SM22a
$2^{\Delta\Delta Ct}$
1500
1000
500
0
Control
GFP
10^8 BMPR2
10^9 BMPR2
c
Anti-GFP Immunoblot of PASMCs
Control
GFP
BMPR2
Control
GFP
BMPR2
50
37
25
20
Anti-BMPR2 Immunoblot of PASMCs
Control
GFP
BMPR2
150
75
50
37
d
rBMPR2 expression vs. SM22a
$2^{\Delta\Delta Ct}$
50
40
30
20
10
0
*
Proximal
Mid
Distal
e
rBMPR2 expression vs. 18s
$2^{\Delta\Delta Ct}$
15
10
5
0
*
*
Control
GFP
hBMPR2

Figure 5-3

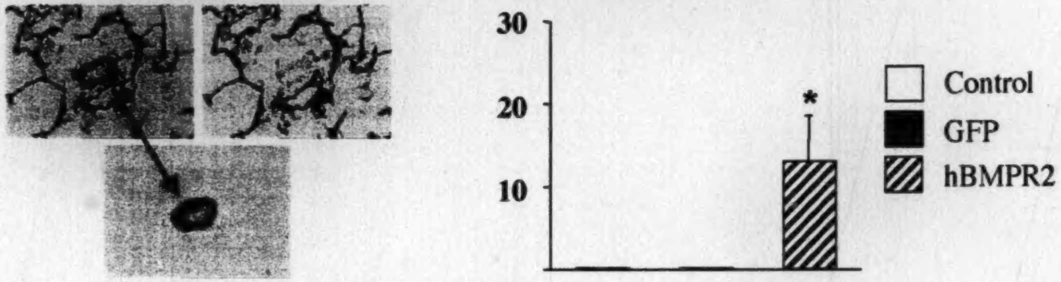
a


Fluorescence
Phase Contrast
Control
Ad-BMPR2

b


Robust hBMPR2 relative expression vs. 18S throughout lung fields

c


Laser Capture microdissection of resistance PAs

Relative hBMPR2 expression vs. 18S in microdissected resistance PAs

Figure 5-4

Invasive Hemodynamics

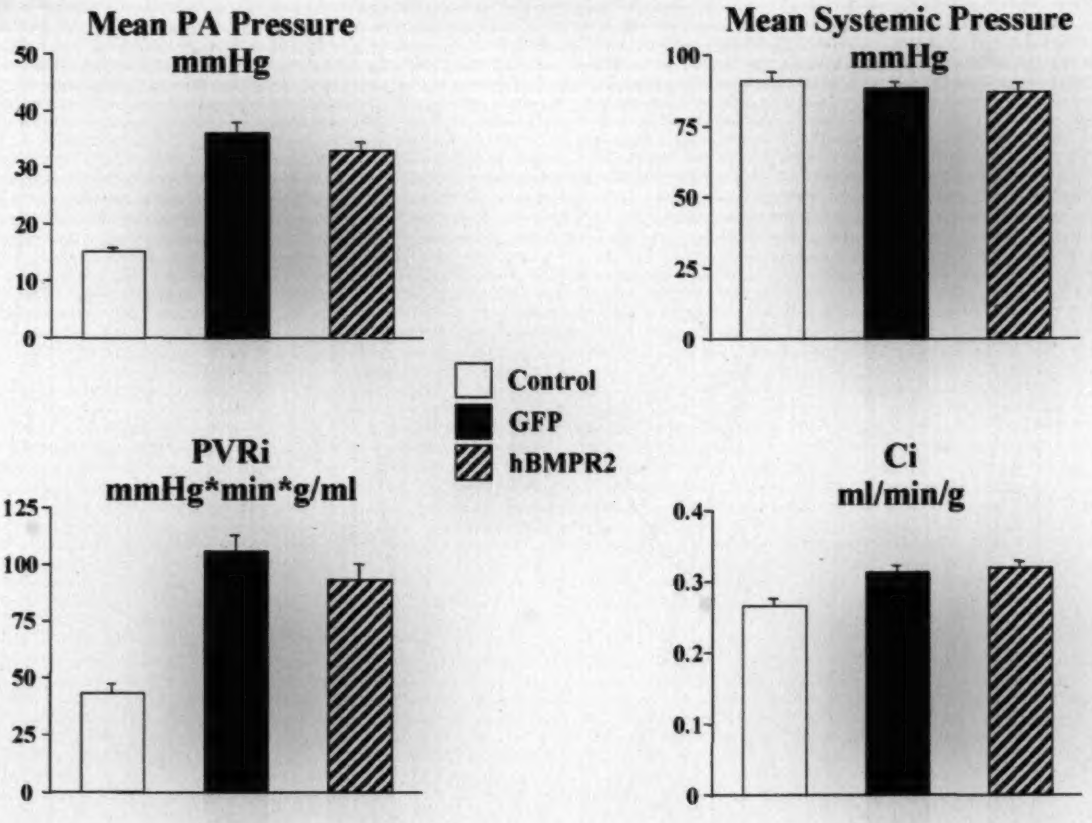


Figure 5-5

Echo Indices and Remodeling

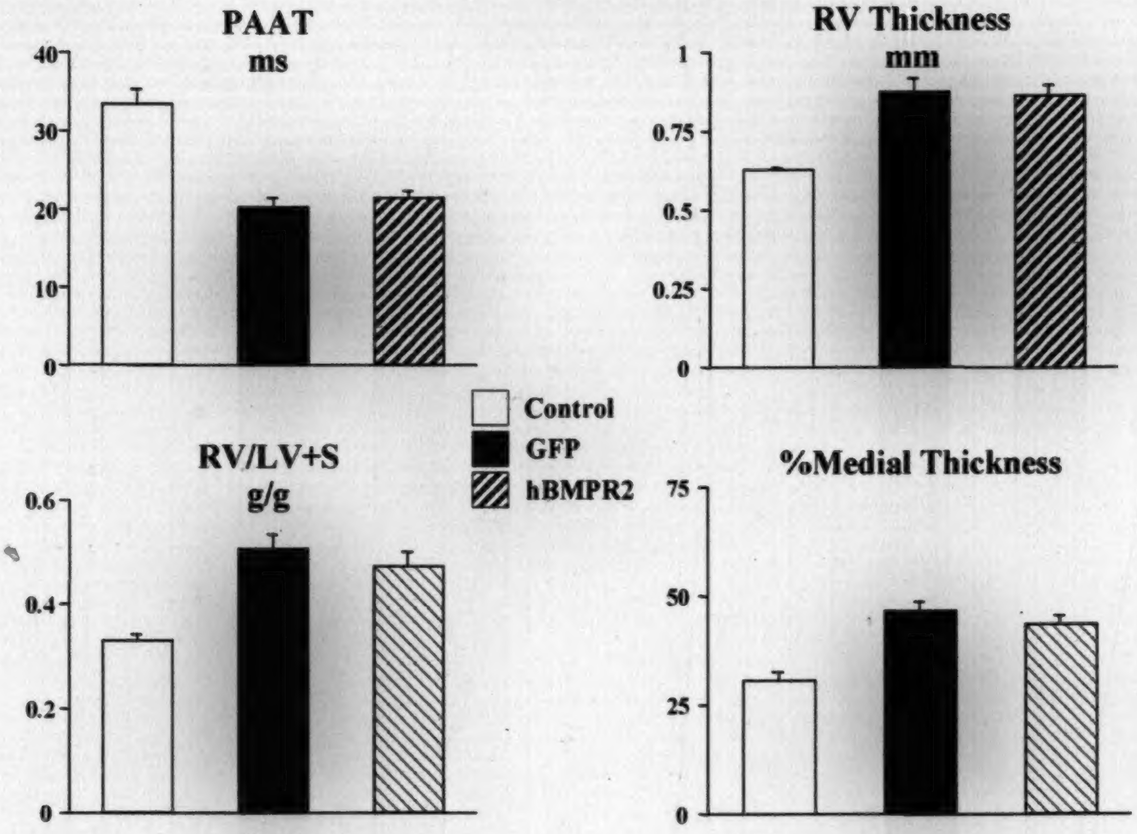
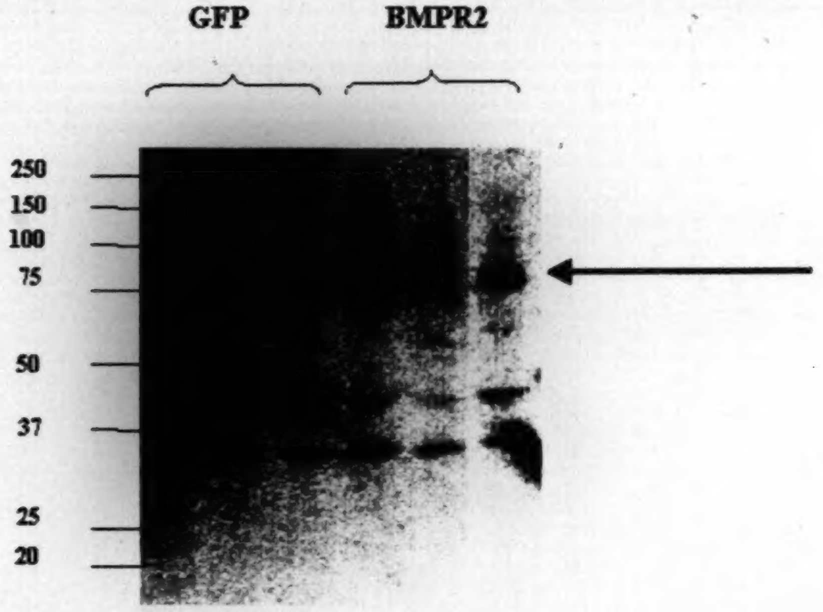


Figure 5-6

BMPR2 protein expression is increased by Ad-hBMPR2 treatment


GFP
BMPR2
250
150
100
75
50
37
25
20

Chapter 6 - Discussion

PAH is a syndrome of elevated pulmonary arterial resistance, caused by a set of disorders with common pathobiological processes that lead to a clinical presentations of dyspnea, chest pain, syncope, and early mortality(1). The 1998 WHO conference in Evian, France, divided all pulmonary hypertensive diseases into 5 categories, later updated in Venice in 2003, that shared similar causes and approaches for therapy. The first category is that of PAH which includes familial and sporadic PPH, or iPAH, as well as PAH associated with collagen vascular diseases, congenital systemic to pulmonary shunts, portal hypertension, drugs and toxins, and persistent pulmonary hypertension of the newborn (PPHN)(14). The definition of PAH thus includes several disorders, grouped more by similarities in clinical presentation and current therapeutic management, as opposed to mechanistic factors(4). As a result, this schema makes unraveling the pathobiology and developing new therapies challenging.

PAH is characterized by elevated pulmonary vascular resistance (PVR), which is due to the processes of vasoconstriction, vessel remodeling, and vessel obstruction due to thrombosis. Important derangements that may be pathogenic have been described at each level of the pulmonary artery. These derangements include abnormal endothelial cell growth and function, with increased endothelial derived vasoconstrictors and mitogens and decreased endothelial derived vasodilators; abnormal growth and proliferation of pulmonary artery smooth muscle cells with abnormal phenotypes, including reduced K+ channel expression; abnormal platelets with decreased levels of serotonin (5-HT) in the platelets and increased levels in the serum; abnormal activation of proteases within the vessel wall, and a procoagulant state(1, 6). Unraveling the links between these seemingly unrelated mechanisms is challenging, but it is apparent most of these abnormalities cause either enhanced PA vasoconstriction, impaired apoptosis, or accelerated growth of the vascular cells that comprise the resistance PA. Perhaps not all mechanisms are important at a given stage of PAH or in a given patient. Furthermore, each mechanism might be by itself insufficient to cause PAH, but several mechanisms acting in concert might be sufficient to cause the disease. A "multiple-hit" theory, involving several genetic and environmental factors, has therefore been proposed(1).

This work comprises four separate investigations that study mechanisms of monocrotaline PAH (MCT-PAH) in rats and experimental therapies. The goal of each study was to target the proliferative vascular remodeling that characterizes PAH, over and above simply vasodilating the pulmonary arterial bed, and to therefore advance therapy over currently available vasodilating approach(2, 5).

Chapter 2 - DCA

In the first study, dichloroacetate (DCA), a metabolic modulator that increases mitochondrial oxidative phosphorylation, was shown to prevent and reverse established monocrotaline-induced PAH (MCT-PAH), significantly improving mortality, and not affecting systemic hemodynamics or causing significant toxicity. DCA depolarized MCT-PAH PASMC mitochondria and caused release of H_2O_2 and cytochrome c, inducing a 10-fold increase in apoptosis within the PA media (TUNEL and caspase 3 activity) and decreasing proliferation (proliferating-cell nuclear antigen and BrdU assays). Immunoblots, immunohistochemistry, laser-captured microdissection-quantitative reverse-transcription polymerase chain reaction and patch-clamping showed that DCA reversed the Kv1.5 downregulation in resistance PAs, although the mechanism by which DCA altered the transcription of Kv1.5 was not determined. In summary, DCA reverses PA remodeling by increasing the mitochondria-dependent apoptosis/proliferation ratio and upregulating Kv1.5 in the media.

An important observation of this study is that the mitochondria of PASMCs have a central role in the maintenance of vascular tone, by releasing H_2O_2 and activating K+ channels, as well as maintaining the balance of apoptosis and proliferation of these cells, by both mitochondrial dependent pathways involving cytochrome c release and by activating K+ channels, reducing intracellular K+, and releasing a tonic inhibition of caspases. To our knowledge, this is the first report of this observation in the literature. PASMC mitochondria potentially link the K+ channel deficiency theory of PAH with the proliferative remodeling seen in PAH, beyond the simple mitogenic effects of increased intracellular calcium, which might in turn be modulated by other concomitant abnormalities, such as 5-HT overload or a genetic preponderance to unregulated PASMC proliferation due to abnormal BMPR2. Furthermore, PASMC mitochondria are attractive as possible targets for effective and selective drug therapy for PAH.

Because DCA is selective for the pulmonary circulation, has a very good safety profile in PAH rats and humans(16), and is orally available, it is a very attractive potential therapy for human PAH, a disease in which effective, simple to deliver, and nontoxic therapies are urgently needed. Based on these data, it is reasonable to propose a clinical trial of DCA in human PAH.

Chapter 3 - Survivin

In the second study, we found that survivin was expressed in the pulmonary arteries (PAs) of 6 patients with PAH and rats with monocrotaline-induced PAH, but not in the PAs of 3 patients and rats without PAH. Survivin is an "inhibitor of apoptosis" protein, previously thought to be expressed primarily in cancer cells. Gene therapy with inhalation of an adenovirus carrying a phosphorylation deficient survivin mutant with dominant-negative properties reversed established monocrotaline-induced PAH and prolonged survival by 25%. The survivin mutant lowered pulmonary vascular resistance, RV hypertrophy, and PA medial hypertrophy. Both in vitro and in vivo, inhibition of survivin induced PA smooth muscle cell apoptosis, decreased proliferation, depolarized mitochondria, caused efflux of cytochrome c in the cytoplasm and translocation of apoptosis-inducing factor into the nucleus, and increased Kv channel current; the opposite effects were observed with gene transfer of WT survivin, both in vivo and in vitro. This work builds upon the work of the first chapter, and strengthens the role of PASMC mitochondria as central controllers of PASMC apoptosis and growth.

An important finding is that the gene therapy did not prevent PAH when given on day one with the MCT, but did reverse established MCT-PAH. This finding might be explained by in part by the lack of survivin expression at that time point, but also the possible effects the gene therapy might have on the endothelium. Endothelial cell apoptosis and dysfunction has been implicated early in the development of PAH(17, 19), and promoting apoptosis in that compartment early in the disease may not be beneficial(8).

Our study further validates the concept of therapies that target the proliferative vascular remodeling of PAH, which is an advance in the field. In addition, our data, together with other similar studies in the literature(12), validates the inhaled gene therapy approach for PAH as being effective, selective, and with minimal toxicity. Although gene therapy for PAH has yet to be validated in humans, our data suggest that it is feasible pending vectors with appropriate profiles of bioavailability and safety. Finally, inhibition of the inappropriate expression of survivin that accompanies human and experimental PAH is a novel specific therapeutic strategy that acts by inducing vascular mitochondria-dependent apoptosis.

Chapter 4 – Rapamycin and Atorvastatin

In the third chapter, we studied combination therapy with rapamycin and atorvastain to attempt to reverse MCT-PAH. Rapamycin had previously been shown to prevent but not reverse rat MCT-PAH(10), while simvastatin, a less potent statin than atorvastatin, had previously been shown to dramatically reverse MCT-PAH by inducing apoptosis of neointimal smooth muscle cells(11). Based on the strength this one animal study, there is a clinical trial based at Imperial College London currently randomizing human patients with PAH to simvastatin or placebo (ClinicalTrials.gov Identifier: NCT00180713). The rationale for combination therapy was that ideally two drugs acting by different mechanisms would act synergistically. Despite high doses of both agents, neither rapamycin nor combination therapy significantly reduced mean pulmonary arterial pressure, echocardiographic indices of pulmonary hypertension, or right ventricular hypertrophy. A weak trend for reduced pulmonary vascular resistance index was observed in both treatment groups. No synergy of the combination was observed. Rapamycin significantly attenuated phosphorylation of P70 S6 kinase in lung tissue of treated animals, confirming adequacy of rapamycin dosing.

It is not clear why these studies were negative, but high doses of both drugs were used and the study power was adequate. These negative findings stress the importance of scientific replication to ensure experimental results are valid prior to proceeding to human clinical trials. Furthermore, this work cautions against assuming a class effect for similar medications. Additionally, the work suggests that a "multiple-hit" might be necessary for therapy, as well as pathogenesis. Rapamycin might be sufficient to slow the development of PAH by inhibiting cell growth, but it is not sufficient to reverse it and other therapies are needed to achieve this. It might be that rapamycin could be a useful adjuvant to a different efficacious therapy, such as DCA, though this is speculative.

Chapter 5 – BMPR2

In this experiment, we studied whether overexpression of BMPR2 in resistance PAs rat MCT-PAH would ameliorate PAH. Gene therapy with an adenovirus encoding human BMPR2, driven by a CMV reporter, was given by the inhalation route and gene and protein expression were confirmed by fluorescence microscopy, qRT-PCR of whole lung samples, and laser capture micro-dissected resistance PAs. Invasive hemodynamic and echocardiographic endpoints of pulmonary hypertension were assessed on day 24. Despite the fact that inhaled Ad-hBMPR2 resulted in robust hBMPR2 expression in all lung lobes and within resistance PAs, it did not improve monocrotaline pulmonary hypertension.

This study confirms the adequacy of inhaled adenoviral gene therapy in generating therapeutic transgene in resistance pulmonary arteries throughout the lung fields. A substantial weakness of this work in its present for is that the downstream signaling of the BMP axis in our treated rats was not dissected. It might be that distal effectors of the pathway were insufficient to modulate an effect of increased BMPR2, or that our human transgene was somehow ineffective at modulating the BMP pathway of the rat. Further work is needed to clarify these issues.

We show that BMPR2 expression in highest in the resistance pulmonary arteries and demonstrate that expression is reduced in experimental PAH. This association is in accordance with observations of human PAH and supports a possible role for reduced BMPR2 expression and function in the development of both MCT-PAH and PAH more generally. On the surface, our results might appear to indicate a lack of a causal role for deficient BMPR2 is monocrotaline PAH. However, our findings are consistent with the evolving notion that PAH usually occurs as a result of multifactorial insults, i.e. that a BMPR2 mutation or deficiency is permissive. For example, mice with a BMPR2 haploinsufficiency have normal pulmonary hemodynamics and vascular structure, even when challenged with chronic hypoxia(3). However with these heterozygous mice were infused with serotonin they had an exaggerated pulmonary hypertensive response. Thus mice with deficient BMPR2 do not spontaneously get severe PAH(3, 18) but rather are more susceptible to PAH(7, 15). The lack of therapeutic efficacy of receptor replacement therapy is also concordant with the literature showing the limited penetrance of PAH in most families with BMPR2 mutations (15-20%)(9, 13). Indeed it is estimated that the lifetime risk of a BMPR2 mutation carrier of developing PAH is only 10%(9).

Future Directions

This work clearly identifies dichloroacetate as a promising therapy for human PAH, and is attractive to pursue this with preliminary human clinical trials of this agent. This work also identifies the importance of PASMC mitochondria, and mitochondria-dependent apoptosis, as potential therapeutic targets in human PAH. It supplies the rationale for future preliminary animal studies of pharmacologic inhibitors of survivin as possible treatments for PAH, as well as more basic studies exploring the role of apoptosis and remodeling of the resistance pulmonary vasculature in PAH. A key unanswered question of this work relates to the observed decrease in Kv channel expression with both DCA and anti-survivin therapies. Experiments designed to identify the control of transcription of the Kv channels and how it is modulated in disease and with treatment are needed to further characterize this pathway. This work adds to the literature of the role of BMPR2 in the development of PAH, and casts doubt on the promise of BMPR2 replacement as a therapeutic strategy for PAH. Further work is needed to dissect the BMPR2 pathway in disease, to see if there are distal effectors that could be targeted instead. Finally, this work casts caution on the use of statins and rapamycin as possible treatments for human PAH. Further animal studies are needed to determine if there is a potential role for these agents, perhaps in combination with other treatments.

Summary

In summary, this work makes several important advances in the development of novel effective therapies that target the proliferative remodeling that characterizes PAH. They include:

1. Identification of the mitochondria as central controllers of K+ channel activation (and therefore vascular tone) and apoptosis (and therefore the balance of apoptosis and proliferation).
2. Identification of DCA as a potential novel therapy for human PAH that promises to be effective, selective, and safe.
3. Identification of increased survivin expression in remodeled resistance PAs in human PAH, and validation of the increased survivin and PASMC mitochondria as potential targets for therapy in human PAH.
4. Validation of inhaled adenoviral gene therapy as a selective and effective approach to deliver therapeutic transgenes to the resistance pulmonary vasculature.
5. Cautionary observations regarding the potential of rapamycin and atorvastatin, and by implication simvastatin, as therapies for human PAH.
6. Added information that indicates that BMPR2 deficiency is likely a permissive by not sufficient mechanism for the development of PAH.

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